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Regulación de la expresión de CD69 y sus efectos funcionales

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

María Teresa Laguna Lobo

Directora

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TRABAJO PRESENTADO POR MARÍA TERESA LAGUNA LOBO
PARA OPTAR AL GRADO DE DOCTOR EN INMUNOLOGÍA

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INFORMA QUE:

El trabajo presentado como tesis doctoral, con el título: "REGULACIÓN DE LA EXPRESIÓN DE CD69 Y SUS EFECTOS FUNCIONALES" por Dña. María Teresa Laguna Lobo, ha sido realizado bajo mi dirección, en el Departamento de Activación Inmunológica del Instituto de Salud Carlos III y, en mi opinión, reúne todos los requisitos necesarios para poder optar al grado de Doctor en Inmunología.

Madrid, 14 de Diciembre de 2012

Al Universo,

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ABREVIATURAS

AP-1	activator protein 1
BAL	Líquido bronco-alveolar / Broncho-alveolar Fluid
BCGF	Factores de crecimiento para células B / B-cell growth factor
BCL	B-cell lymphoma protein
bp	Pares de bases / Base pair(s)
CAIA	Artritis inducida por anticuerpos anti-Colágeno / Collagen Antibody-Induced Arthritis
ChIP	Inmunoprecipitación de cromatina / Chromatin Immunoprecipitation
ChIP-seq	Secuenciación de inmunoprecipitación de cromatina / Chromatin Immunoprecipitation sequencing
CIA	Artritis inducida por Colágeno / Collagen-induced arthritis
ConA	Concanavalina A / Concanavalin A
CREB	cAMP response element-binding protein
DC	Célula Dendrítica / Dendritic Cell
DN	Doble(s) negativo(s) / Double-negative
DP	Doble(s) positivo(s) / Double-positive
EGR	Early Growth Response proteins
Elk	E-twenty six-like factor
Ets	E-Twenty Six
FACS	Citometría de flujo / Flow Cytometry
Fc	Fracción constante de Inmunoglobulinas / Constant fraction of Immunoglobulin
fMLP	N-formyl-methionine-leucine-phenylalanine
FOX	Forkhead Box (factor)
FoxP3	Forkhead box P3 factor
FV	Friend Virus
GABPA	GA-binding protein alpha chain
GFP	Proteína verde fluorescente / Green Fluorescent Protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hCD69	CD69 de ser humano / Human CD69
HS	Sitio de hipersensitividad / Hypersensitivity Site
IFN	Interferón / Interferon
Ig	Inmunoglobulina / Immunoglobulin
IL	Interleuquina / Interleukin
IRES	Sitio interno de entrada del ribosoma / Internal Ribosome Entry Site
IRF	Factor de respuesta a interferón / Interferon response factor
Jnk3	Quinasa Janus 3 / Janus kinase 3
KO	Knock-out
LEF-1	Factor de unión amplificador de linfocitos / Lymphoid enhancer binding factor
LN	Ganglios linfáticos / Lymph Node
LPS	Lipopolisacáridos / Lipopolysaccharides
mCD69	CD69 de ratón / Mouse CD69

MCP-1	Monocyte chemoattractant protein-1
MFI	Intensidad de fluorescencia media / Mean fluorescence intensity
miRNA	micro-RNA
MLR	Reacción linfocitaria mixta / Mixed lymphocyte reaction
mRNA	RNA mensajero / messenger RNA
NFAT	Nuclear factor of activated T-cells
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
Oct(-1)	Octamer transcription factors (1)
OVA	Ovoalbúmina / Ovalbumin
PBL	Linfocitos de sangre periférica / Peripheral blood lymphocytes
PHA	Fitohemaglutinina / Phytohaemagglutinin
PKC	Protein-kinase C
pLN	Ganglios linfáticos periféricos / Peripheral lymph nodes
PMA	Phorbol 12-myristate 13-acetate.
PPD	Mezcla proteica de antígenos de *Mycobacterium sp.* / Purified protein derivative
PWM	Lectina de Phytolacca americana / Pokeweed mitogen
RAG	Recombination activation gene
RE	Enzima de restricción / Restriction enzyme
RUNX	Runt-related transcription factor
S1P	Sphingosine-1-phosphate
S1P1	Sphingosine-1-phosphate receptor 1
SAC	Proteína A de *Staphylococcus aureus* cepa Cowan I / *Staphylococcus aureus* protein A
siRNA	RNA de interferencia pequeño / Small interfering RNA
SP	Single-positive
SRF	Serum Response Factor
TCR	Receptor de células T / T-cell receptor
TF	Factor de transcripción / Transcription factor
TFBS	Sitios de unión a factores de transcripción / Transcription Factor Binding Site
TFF3	Trefoil factor-3
TG	Transgénico / Transgenic
TGFβ	Factor de crecimiento transformante beta / Transforming growth factor beta
Th1	Población T ayudadora 1 / T helper 1 population
Th17	Población T ayudadora 17/ T helper 17 population
Th2	Población T ayudadora 2 / T helper 2 population
ThPOK	T-helper-inducing POZ/Krüppel-like factor
TNFα	Factor de necrosis tumoral alfa / Tumor necrosis factor alpha
Treg	Células T reguladoras / Regulatory T cells
TT	Tóxina tetánica / Tetanic toxin
WT	Fenotipo salvaje / Wild-type

INTRODUCCIÓN

1. REGULACIÓN DE LA EXPRESIÓN GÉNICA

La actividad biológica está regulada por múltiples señales que modulan la actividad proteica. La cantidad de proteína funcionalmente activa que acaba en su localización final está regulada por diferentes vías:

1) A nivel transcripcional: Entre los mecanismos que regulan la transcripción está la conformación de la cromatina y la interacción de elementos reguladores del DNA con factores de transcripción. La descondensación y/o apertura del DNA es a su vez regulada por modificaciones de las histonas, y/o por metilación del DNA. Es lo que se denomina regulación epigenética, y existen numerosos estudios que revelan su importancia en biología (4), como en evolución (5) o en cáncer (6).

2) A nivel de disponibilidad de RNA mensajero (mRNA): La velocidad de transcripción y degradación del mRNA, junto con la presencia de secuencias ricas en AU o de unión a micro-RNAs (miRNAs) y RNAs de interferencia (siRNAs) son los mecanismos más conocidos que regulan la cantidad de mRNA accesible para ser traducido y transformado en proteína.

3) A nivel post-traduccional: las modificaciones post-traduccionales, la interacción con otras proteínas estabilizadoras (chaperonas) o que faciliten la degradación (complejo ubiquitina), el transporte desde el núcleo al citosol o a la membrana celular a través del Golgi, retículo endoplasmático, vesículas, etc., son mecanismos que regulan la cantidad de proteína funcionalmente activa.

Globalmente, se trata de un proceso extremadamente complejo, aún sin considerar la simultaneidad de señales y el efecto entrecruzado de ellas, en un sistema con múltiples tipos celulares y múltiples tejidos.

A continuación, se van a describir las áreas de la regulación de la expresión génica que se tratan en esta tesis para el estudio de CD69:

Grado conformacional de la cromatina

Para que un gen pueda transcribirse, su DNA tiene que estar parcialmente descondensado (eucromatina) y libre de nucleosomas, lo que se denomina DNA "abierto". Para ello, el DNA debe estar no metilado ("de-metilarse" en el caso de que lo estuviera) y las histonas, que forman los nucleosomas, cambiar su conformación reguladas por diferentes señales (revisado en (7-9)). Estas regiones son la que interaccionan

con proteínas reguladoras de la transcripción, y son sensibles a digestión por nucleasas, como la nucleasa de *Micrococcus*, la Exonucleasa II, etc.; pero la más empleada y testada en genomas de mamífero es la DNasa I

Las zonas accesibles no sólo son aquellas donde se transcribe el gen, sino también aquellas que se han identificado como zonas reguladoras *cis*, que pueden englobar Amplificadores de la transcripción, Silenciadores, Aisladores, Promotores, Terminadores, Orígenes de replicación, Sitios de unión de Topoisomerasa, Sitios de recombinación, Telómeros o Centrómeros (revisado en (10, 11)) (Fig. IN-1). Hay numerosas evidencias de que se encuentran en esta conformación abierta cuando están activas empleando ensayos de hipersensibilidad a DNasa I; por ejemplo, para CD4 (12), CD8 (13), la interleuquina 4 (IL-4) (14), el receptor de células T delta (TCRδ, *T-cell receptor delta*) (15), etc.

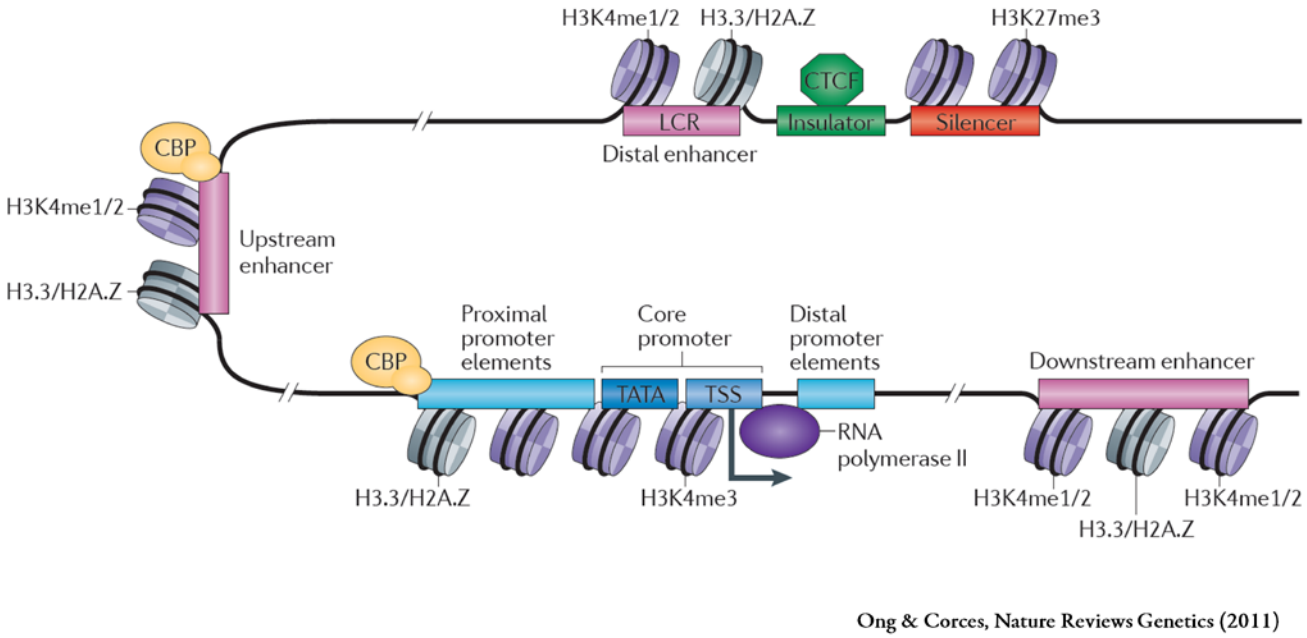

Fig. IN. 1. Elementos de regulación de la transcripción en metazoos. El promotor suele estar compuesto por núcleo (*core*), elementos proximales y elementos distales. La transcripción puede estar regulada por múltiples amplificadores (*enhancers*) que podrían o no estar intercalados con elementos silenciadores (*silencers*) y elementos aisladores (*insulators*) que se unen a proteínas como el factor de unión a CCCTC-(CTCF). En los elementos amplificadores distales en ocasiones se encuentra una región reguladora de *locus* (LCR, *locus control region*), que regularía la expresión de diferentes genes próximos en conjunto. Se ha observado en estudios a nivel genómico que la mayoría de los amplificadores presentan una cromatina con características únicas, como la unión de histonas alternativas y marcas de histonas específicas. Además, también se caracterizan por la unión de la proteína CBP (*CRE binding protein*). *H3K4me1/2*, mono o dimetilación de la lisina 4 en la histona 3; *H3K4me3*, trimetilación de la lisina 4 en la histona 3; *H3K27me3*, trimetilación de la lisina 27 en la histona 3; *H3.3/H2A.Z*, variantes de las histonas H3.3 y H2A.Z; *TATA*, secuencia núcleo de DNA 5′-TATAAAA-3′; *TSS*, sitio de inicio de la transcripción (*transcription start site*).

Regulación mediante factores de transcripción

En la transcripción, la importancia de estas zonas reguladoras en *cis* reside en su capacidad para unir factores de transcripción que modulen este proceso. Según el efecto que provoquen, las zonas a las que se unen estos factores pueden ser (además del promotor que activa la trasncripción) Amplificadores (*Enhancers*), Silenciadores (*Silencers*), Aisladores (*Insulators*), o cumplir más de una de estas funciones dependiendo de la(s) proteína(s) que unan.

Los Amplificadores se han descrito como fundamentales en la función biológica, por ejemplo en evolución (16-19) o en evolución del desarrollo (20-22). En la función inmunológica, aparte de los ya comentados en CD4, CD8, TCRδ e IL-4, se han descrito algunos como en el locus Th2 (*T helper 2*) (23), del gen de FoxP3 (*Forkhead box P3 factor*) (24), o de Pax5 (*Paired box protein 5*) (25), cuyas alteraciones conllevan grandes deficiencias en la respuesta inmune. En la actualidad, se consideran tres modelos de amplificación según la

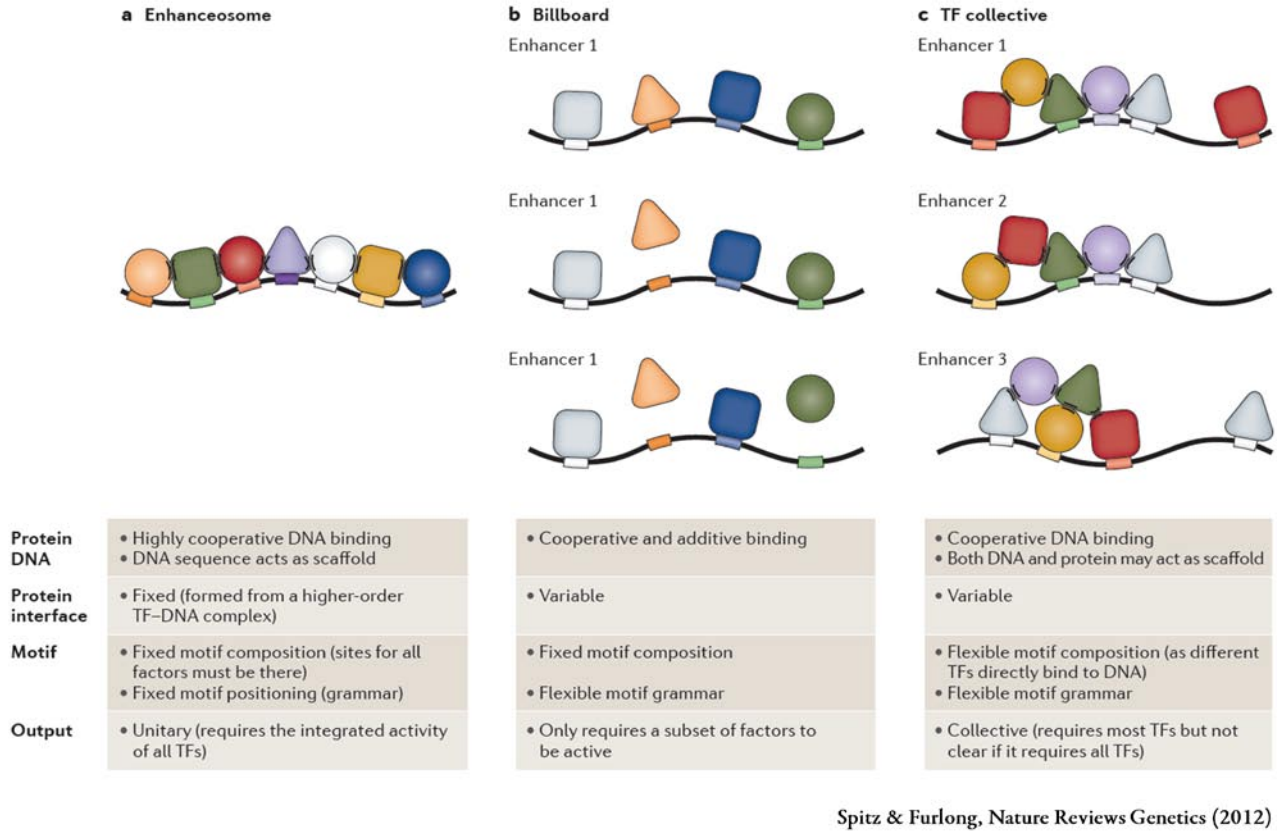

Fig. IN. 2. Modelos teóricos vigentes para la acción de los amplificadores sobre la expresión génica. (a) Modelo de complejo de estructura fija o *Enhanceosome,* donde todos los factores de transcripción son necesarios para la ocupación y la activación del amplificador (1). (b) Modelo de complejo de composición de factores variable pero estructura de motivos fija (*Billboard*), donde la unión de cada factor no es necesaria pero sí cooperativa, pudiendo estar activos unos elementos diferentes en cada momento (2). (c) Modelo de unión a diferentes amplificadores, donde el mismo grupo de factores se pueden unir de diferente manera a distintas secuencias amplificadoras, regulando la transcripción como un conjunto (3).

participación de los diferentes factores de transcripción (26) (Fig. IN.2):

- Mediante la unión de complejos multiproteicos de estructura fija que actúan como una sola unidad (*Enhanceosome*). En este caso una mutación en el amplificador o la ausencia de uno de los factores inhiben significativamente el efecto inductor de la transcripción.
- Mediante la unión de complejos de composición flexible pero con composición de motivos de unión fijos (*Billboards*). No es necesaria la presencia de todos los factores para su función, permitiendo la modulación de su efecto.
- Mediante la unión de complejos de factores a distintos amplificadores (*Transcription Factor collective*). En este caso, un mismo grupo de factores de transcripción se puede unir a distintas secuencias reguladoras de diferentes formas, pudiendo interaccionar factores determinados con un amplificador y otros diferentes con otro adoptando distintas conformaciones.

Para los silenciadores, también se han propuesto mecanismos de represión, que pueden implicar la unión de factores represores a secuencias amplificadoras o bien a secuencias específicas de represión (revisado en (27)). Referente a los aisladores, los últimos estudios desvelan una regulación compleja, pudiendo inhibir o favorecer la transcripción (revisado en (28)). Ejemplos estudiados en el campo de la Inmunología son el caso del silenciador de CD4 (29) o aisladores en la zona del complejo de histocompatibilidad de clase II (30).

1. 1. HERRAMIENTAS DE ESTUDIO DE LA REGULACIÓN DE LA EXPRESIÓN GÉNICA

Dentro de la gran diversidad de aproximaciones teóricas y experimentales para el estudio de la regulación génica, para el desarrollo de esta tesis se emplearon los siguientes que se describen a continuación:

Estudios in vitro

i. Transfección con reporteros de luciferasa

La detección de los niveles transcripcionales mediante luminiscencia está ampliamente extendida, en concreto mediante el uso de luciferasas, grupo de enzimas oxidativas que catalizan reacciones productoras de luz, distintas de las fotoproteínas (31). Para ello, se dirige la expresión del cDNA de la luciferasa mediante

promotores u otras secuencias reguladoras, clonándolos en el vector de expresión en la posición adecuada. Se añade el sustrato correspondiente, de tal forma que la cantidad de luz emitida es proporcional a la cantidad de tránscrito, lo que permite comparar el efecto de distintas regiones de DNA en la regulación de la transcripción del gen de interés.

Esta técnica se ha empleado satisfactoriamente para el estudio de elementos reguladores *cis*, como en los genes de la integrina β7 (32), IgH (33, 34), TFF3 (35), IL-2 (36), o CD40L (37)

ii. Mutagénesis Dirigida

Mediante PCR con oligo mutado en su parte central, digestión mediante enzima de restricción del plásmido bacteriano y transformación, se puede obtener de manera sencilla un vector con una mutación específica en la posición deseada (ver Fig. I.M.2, Sección I.M) (38).

De esta manera, se han podido detectar elementos fundamentales en la transcripción de moléculas como IgH (39), Jnk3 (40), B7-H1 (41) o TRIM22 (42).

Líneas de ratón modificadas genéticamente

Existen diferentes tipos de líneas de ratón empleadas para el estudio de la regulación de la expresión génica:

a) LÍNEAS *KNOCK-OUT* (KO): La modificación introducida en estos ratones consiste en sustituir una parte necesaria del gen para su expresión, normalmente varios exones e intrones, por una secuencia para poder seleccionar los recombinantes positivos. Esto provoca la eliminación de la expresión de la proteína. También se emplean para sustituir zonas reguladoras en lugar del propio gen.

b) LÍNEAS CONDICIONALES O *KNOCK-IN* (KI): en este caso, el gen no se elimina del DNA, si no que se añaden secuencias para su eliminación que respondan ante determinados estímulos o en determinados linajes celulares.

c) LÍNEAS *BAC-IN*: aquí se introduce un cromosoma artificial bacteriano (BAC, *Bacterial Artificial Chromosome*) de 100 a 200 kb que contiene el gen de estudio y las zonas no codificantes tanto en 5' como en

3', presuponiendo entonces que están presentes todas (o al menos la mayor parte) las zonas reguladoras de la transcripción del gen. Permite modificaciones en estas regiones para observar su efecto en la expresión en el alelo del BAC, pero no en los endógenos.

Aplicaciones informáticas

En la última década han surgido multitud de herramientas informáticas para facilitar el estudio de la regulación génica, como los programas de análisis de secuencias y sus características, o las bases de datos con todo tipo de información sobre regulación. Muchas de ellas se encuentran disponibles en la red, facilitando la distribución y la integración del conocimiento regulatorio en toda la comunidad científica. Las más empleadas para la elaboración de esta tesis son:

i) Vista Genome Browser (43, 44): Permite la visualización comparativa de la conservación de secuencias entre especies, indicando las zonas con alta conservación no codificantes, exones, intrones, UTRs, y la extracción de las secuencias para trabajar con ellas fuera de línea (http://pipeline.lbl.gov/cgi-bin/gateway2). Para ello, tiene una base de datos con gran cantidad de secuencias completas de genomas de procariotas y eucariotas.

Permite el acceso mediante enlaces a otras herramientas de la plataforma *VISTA Tools*, como *rVISTA*, que localiza secuencias reguladoras en una secuencia de DNA, *mVISTA* y *VISTA Point*, que permiten la comparación de distintas secuencias.

Además, tiene la opción de visualizar las secuencias de la base de datos en el navegador de la Universidad de California-Santa Cruz (*UCSC Browser*), que a su vez presenta multitud de utilidades:

ii) UCSC Browser: Al igual que la plataforma anterior, tiene una base de datos con multitud de genomas, pero además, tiene integrados gran cantidad de datos provenientes de estudios genéticos. Entre ellos, hay datos de estudios sobre regulación génica, tales como hipersensibilidad a DNasa I, inmunoprecipitación de cromatina (ChIP, *chromatin immunoprecipitation*) para diversos factores de transcripción, marcas de acetilación de histonas, etc. Algunos de estos datos de estudios sobre muestras humanas provienen del consorcio ENCODE.

iii) Genomatix: Portal de internet de acceso limitado que posee potentes herramientas para el análisis génico. Una de ellas, llamada *Genomatix DiAlign*, permite obtener sitios de unión de factores de transcripción conservados (*conserved TFBS*) en distintas especies. Posee una base de datos muy amplia de factores de transcripción agrupados en familias y subfamilias, así como una herramienta de búsqueda de bibliografía (de libre acceso) bien estructurada.

iv) MGI Portal: Esta base de datos contiene la información relacionada con todas las líneas de ratón transgénicas producidas hasta la actualidad. En el campo de la regulación génica es útil porque permite consultar el fenotipo de ratones KO, KI, BACin de cualquier gen de interés, la expresión en líneas celulares, su posible función, su homología con ortólogos, etc.

La localización de zonas conservadas no codificantes (CNS, *Conserved Non-coding Sequence*) mediante estas herramientas ha sido empleada en numerosos casos dentro del estudio del Sistema Inmune, como en el caso del interferón gamma (IFNγ) (45), cuyo gen necesita la zona reguladora CNS-22 para expresarse en células T; o el de FoxP3 (24) donde cada CNS dirige unos patrones de expresión diferentes. En ambos casos se empleó *Vista Genome Browser* para localizar estos CNS, al igual que hizo nuestro grupo previamente en el genoma de ratón (46) y humano.

2. CD69: RECEPTOR RELEVANTE PARA EL SISTEMA INMUNE

CD69 se describió inicialmente en el ser humano como una proteína de membrana fosforilada con 2 subunidades de 28 kb y 32 kb, unidas por puentes disulfuro, y de expresión temprana en linfocitos (EA1, del inglés *Early activation 1*). También se le denominó Leu-23 (47), AIM (48) y MLR3 (49) debido a los estudios de diferentes grupos que partieron desde fuentes diferentes; hasta que finalmente se decidió unificar la denominación en CD69 en la reunión *Fourth Workshop on Human Leukocyte Differentiation Antigens*.

CD69 pertenece a la familia de receptores con dominios tipo lectina de clase C (*C-type lectin-like receptors*), cuya característica general es que unen carbohidratos de manera específica y dependiente de Calcio. Se trata de una familia con numerosos miembros y subfamilias, cuyas funciones están altamente relacionadas con el sistema inmune, por ejemplo, mediando en adhesión y migración o participando en el reconocimiento de patógenos (50).

La subfamilia a la que pertenece CD69 es tipo-receptor NK (*Natural Killer receptor-like*), y sus miembros se caracterizan por ser receptores transmembrana tipo II (con el extremo C-terminal extracelular), y porque el dominio tipo lectina carece de los residuos para la unión de Calcio (51) . Estos dominios similares a lectinas se denominan CTLDs (*C-type lectin-like domains*), y más concretamente en esta subfamilia, NKDs (*NK domains*). Los NKDs pueden formar *in vivo* hetero- u homo-dímeros, como es el caso de CD69 (52, 53). Estos y otros estudios (54) no han encontrado indicios de unión a carbohidratos dependiente de Ca^{2+}, sin embargo existen otros que establecen esa interacción (55-58).

Se desconoce su ligando fisiológico, aunque se han llevado a cabo diversos intentos por encontrar moléculas con alta afinidad por CD69. Se han establecido como ligandos N-acetil-hexosaminas (59) y compuestos sintéticos denominados calixarenos (60). Estos últimos parecen inhibir la apoptosis de células citotóxicas mediada por ligandos de células tumorales, lo cual permitiría modular la acción de CD69 para posibles usos terapéuticos (61-63).

2.1. EXPRESIÓN TISULAR

Numerosos trabajos han estudiado la expresión del receptor CD69 en las membranas celulares, en los cuales se ha observado una expresión restringida al linaje hematopoyético.

En linfocitos humanos, se ha observado que la expresión de CD69 en la subpoblación CD3 *bright* de timocitos (64) y linfocitos T y B de sangre y tejidos (65, 66), siendo inducible *in vitro* empleando ésteres de forbol como PMA (*phorbol 12-myristate 13-acetate*), lectinas (ConA, PWM, PHA)[1], antígenos (TT, PPD)[1], por reacción linfocitaria mixta y anti-IgM más factores de crecimiento para células B (BCFG) (67), anti-CD3 (49), anti-CD2, anti-CD28 (68) o incluso agentes estresantes, como choque térmico (68) o la irradiación con rayos gamma (69).

En neutrófilos de sangre periférica humana, no hay expresión aparente de CD69 en la membrana, aunque sí inducible con PMA (70-72) y otros estímulos como fMLP (*N-formyl-methionine-leucine-phenylalanine*), el factor estimuladore de colonias de granulocitos/macrófagos (GM-CSF), IFN-γ ó IFN-α (72). Algunos trabajos proponen que existe la proteína preformada en el citosol (70, 71), mientras que otros lo desmienten (72).

La expresión de CD69 en basófilos de sangre venosa humana también es inducible *in vitro* mediante la adición de IL-3, pero no de otros estímulos como IL-4, IL-5, GM-CSF, IFN-γ, eotaxina o la proteína quimoatractante de monocitos 1 (MCP-1) (73). *In vivo*, CD69 se expresa en mayor medida en basófilos extraídos del líquido broncoalveolar que en sangre en individuos asmáticos (73).

En eosinófilos de origen venoso, se induce la expresión de CD69 tras estímulo con IL-5 (73). En estas células, pero obtenidas de sangre periférica, GM-CSF actúa también como estimulador (71), además de IL-5, IL-5 más eotaxina, lipopolisacáridos (LPS), fMLP (71), fosfolipasa A2 (74) e IL-3, pero no usando PAF (*Platelet-Activating Factor*) (75). Al igual que en neutrófilos, se detectó la presencia de CD69 intracelular (71). También se ha encontrado su expresión *in vivo* en eosinófilos del líquido broncoalveolar en enfermos de asma (75).

En monocitos existen evidencias de una expresión constitutiva en sangre periférica humana (76), pero no de que existan formas intracelulares (71). No obstante, en macrófagos de ratón derivados de médula ósea, no se

[1)] Nombre completo en Sección Abreviaturas.

ha encontrado una expresión constitutiva, pero sí inducible por LPS + IFN-γ ó TFN-α + IFN-γ; pero no por PMA (77).

En células precursoras mieloides también se ha detectado la expresión de CD69 en la membrana. Es el caso de precursores de eosinófilos derivados de sangre de cordón, donde hay una expresión constitutiva baja pero inducible con IFN-γ (78). También se encontró la expresión constitutiva en promielocitos extraídos de médula ósea o provenientes de la línea celular HL-60 (70).

También se ha hallado su expresión constitutiva en plaquetas (79) y células de Lagerhans (80), en estas últimas pudiendo mantener su expresión añadiendo IFN-γ recombinante.

Respecto a la velocidad de expresión tras estímulo, al caracterizar CD69 por primera vez se observó que es muy rápida en linfocitos, apreciándose de su expresión en el intervalo de una hora (67). Por contra, en distintos linajes mieloides, el máximo de expresión de CD69 se alcanza en un tiempo aproximado de 24 horas, siendo posterior su expresión a las de otras moléculas inducidas tras la activación (73, 74, 77, 78).

En resumen, los leucocitos se pueden dividir en dos subgrupos: los que expresan CD69 constitutivamente, como plaquetas y células de Lagerhans; y los que presentan una expresión transitoria, como los timocitos en desarrollo. Dentro del último grupo, pueden mostrar una inducción rápida, como linfocitos T, B y células NK (*natural killer*); o más lenta, como granulocitos y monocitos/macrófagos.

2.2 FUNCIÓN

La función de biológica de CD69 se lleva estudiando desde finales de la década de los 80. Debido a los resultados obtenidos desde entonces, se han propuesto diferentes sunciones en dos aspectos del funcionamiento del sistema inmune: como regulador de la respuesta, y como regulador del tráfico leucocitario.

a) REGULADOR DE LA RESPUESTA INMUNE

i. Molécula coestimuladora

Tras los primeros estudios *in vitro* sobre CD69 realizados en células humanas, se propuso como papel de CD69 el de molécula coestimuladora, debido que se observaban efecto estimuladores de las funciones características de cada leucocito.

En linfocitos T tratados con PMA y anticuerpos anti-CD69, se observó la inducción de la expresión de IL-2, IFNγ (48, 81), IL-2R/CD25 (82, 83), el factor de necrosis tumoral alfa (TNFα) (84), y en ocasiones, un efecto proliferativo (82, 83). Entrecruzando CD69 pero sin estimular la protein-quinasa C (PKC) mediante PMA, se produce un aumento de calcio intracelular, pero no el resto de efectos descritos (82).

El efecto es el mismo en linfocitos B (66), donde la estimulación conjunta induce la misma proliferación que utilizando PMA y anti-IgM (inmunoglobulina M). Sin embargo, el entrecruzamiento de CD69 inhibe la proliferación en células B estimuladas previamente con antígeno SAC (Proteína A de *Staphylococcus aureus cepa Cowan I*), salvo si el estímulo ocurre antes de 48 h del entrecruzamiento.

En monocitos humanos, el entrecruzamiento de anticuerpos anti-CD69 conlleva la entrada de flujo de Ca^{2+}, la activación de las vías ciclooxigenasa y lipooxigenasa, una elevada producción de óxido nítrico (NO), y finalmente la inducción de citotoxicidad frente a líneas tumorales de ratón (76). En neutrófilos se produce la entrada de calcio tras estímulo con PMA y tras el entrecruzamiento con anticuerpo, aumenta la secreción de lisozima, estimulándose la exocitosis de gránulos (citólisis) (70). Una activación similar se ha descrito en plaquetas, donde el entrecruzamiento de anti-CD69 produce la activación de la vía de la ciclooxigenasa y la agregación plaquetaria (79).

Por otra parte, diversos tratamientos con anticuerpos anti-CD69 han constatado la apoptosis de monocitos humanos previamente activados con LPS (85), aunque parece una consecuencia de una sobre-activación de estas células, ya que por sí sólo anti-CD69 no es capaz de inducir apoptosis (86). En el caso de eosinófilos activados con citoquinas, anti-CD69 sí produce apoptosis de manera rápida, sugiriendo un mecanismo de eliminación acelerada de este linaje celular (87). Además, se observó la producción de IL-1b en monocitos activados por células T tratadas con anti-CD69, que estimularía la destrucción celular (88).

ii. Molécula inhibidora de la respuesta proinflamatoria

Cuando aparecieron los primeros modelos *in vivo* sobre la función de CD69 se descubrió una posible función reguladora frente a inflamación, distinta a la propuesta a partir de los datos obtenidos *in vitro*.

- *Respuesta inmune en líneas CD69-KO*

Utilizando dos líneas de ratón CD69 -/- y tratamientos con anticuerpos anti-CD69 se analizó la respuesta inmune en diferentes modelos. El primer ratón CD69 *knock-out* (de aquí en adelante, "CD69KO1") se generó mediante deleción de los exones II, III, IV y la parte codificante del exón V de la secuencia génica, observándose un fenotipo normal salvo la apreciación de un ligero aumento en la población pre-B $B220^{high}$ IgM^{neg} respecto al ratón *wild-type* (WT) y una respuesta con mayores niveles de IgG2a e IgM tras diferentes inmunizaciones dependientes y no dependientes de células T (89). Se generó posteriormente una segunda línea de ratones deficientes en CD69 ("CD69KO2"), donde el fragmento suprimido consiste en el promotor, el primer exón y parte del primer intrón (90).

En modelos inflamatorios, por ejemplo diferentes formas de artritis, ambos ratones KO presentaron respuestas diferentes. Mientras la línea CD69KO1 mostraba una respuesta similar al WT en los modelos de artritis inducida por anticuerpos anti-colágeno (CAIA, *Collagen Antibody-Induced Arthritis*) (91) y menor producción del factor de crecimiento transformante beta (TGFβ) en artritis inducida por colágeno (CIA, *Collagen Induced Arthritis*) (92), el ratón CD69KO2 resultó estar más protegido frente a CAIA (90). En tratamientos con anticuerpos, empleando anti-CD69 2.2, que mimetiza el fenotipo CD69 -/- , no se apreció diferente afectación en la línea KO; y sí un aumento de la expresión de moléculas inflamatorias como IL-1b, MCP-1 e IL-6 (91).; mientras que el tratamiento con anti-CD69 2.3 provocó efectos opuestos (93).

En modelos de infección se han estudiado los modelos de *Listeria monocytogenes* e infección retroviral por *Friend Virus* (FV). En el caso de *Listeria*, bacteria intracelular que provoca una respuesta ayudadora tipo 1 (Th1, *T helper 1*), el ratón CD69KO1 es más susceptible a la infección debido al daño tisular generado por una mayor expresión de citoquinas Th1; mientras que el ratón RAG2 -/- (gen de activación de la recombinación 2, *recombination activating gene 2*) CD69KO1 -/- , elimina la infección de manera más eficaz (94). En la misma línea se observó que la infección por FV provoca un aumento de T CD4+ CD69+, población que posee características reguladoras (95).

Respecto a la respuesta antitumoral, en el ratón CD69KO1 es más eficaz que el ratón WT frente a tumores MHC-I deficientes, basado principalmente en una mayor actividad citolítica de células NK y T, al expresar más citoquina MCP-1 y menos TGFβ (96). Esta respuesta exacerbada también es posible si se trata un ratón WT tratado con anticuerpo anti-CD69 2.2, que internaliza la expresión de CD69 en todas las células que lo expresan pero no las elimina (96, 97).

En experimentos *in* vitro, se ha descrito un aumento de la secreción de la citoquina reguladora TGF-β1 en células NK y linfocitos CD3+ (96), en esplenocitos tratados con ConA y leucocitos sinoviales de ratón afectado por CIA (92) mediante entrecruzamiento de CD69 *ex vivo* .

También surgieron resultados contradictorios utilizando el modelo de asma alérgico con inflamación de las vías aéreas inducida por péptido de ovoalbúmina (OVA), donde el ratón CD69KO1 muestra una respuesta alérgica aumentada (mayor reclutamiento de eosinófilos y mayor expresión local de citoquinas Th2 y Th17) (98) y en la línea CD69KO2, disminuye el número de eosinófilos y la expresión de citoquinas Th2 en el líquido broncoalveolar (99). En el modelo de hipersensibilidad por contacto empleando como agente alergénico Oxazolona, el ratón CD69KO1 mostró mayor severidad clínica y una mayor expresión de citoquinas Th1 (98).

- *CD69 y Th17*

En ambos modelos de alergia, se ha establecido recientemente que las células Th17 juegan un papel importante como citoquina proinflamatoria (100, 101). El primer trabajo que observó una relación entre CD69 e IL-17 fue el de Srinivasan y col. (102), donde la señalización por el receptor de esfingosina-1-fosfato 1 (S1P1, *sphingosine-1-phosphate receptor 1*) en el modelo de diabetes en ratón (NOD, *non-obese diabetic mice*) aumenta la expresión de CD69 y disminuye la de IL-17 en las células T CD4+. Posteriormente se vio en el ratón transgénico OTII CD69-deficiente (derivado de CD69KO1) un aumento de la población Th17 en los ganglios linfáticos (LNs, *lymph nodes*) próximos, y también en el modelo de CIA en CD69KO1 (103). En el modelo inmune de miocarditis autoinmune experimental (EAM, *experimental autoinmune miocarditis*), se encuentra también un mayor número de células del linaje Th17 (104).

La diferenciación *in vitro* de T CD4+ de CD69KO1/OT-II hacia Th17 provoca una mayor expresión de IL-17 y menor de IFNγ. Además, se observó una interacción de la cola citoplasmática de CD69 con la quinasa Janus 3 (Jak3) y del transductor de señales y activador de la transcripción 5 (Stat5, *Signal transducer and activator of transcription 5*), responsables de la inhibición de la expresión del receptor RORγt, característico del linaje Th17 (103).

- *CD69 y las células T reguladoras*

Recientemente, se ha establecido la relación entre la expresión de CD69 y la función reguladora en linfocitos T. En modelos de tumores hepático, pulmonar y de melanoma en ratón (105), se demostró la existencia de una población reguladora (de CD4+ efectoras) dentro de la población CD4+ CD69+ CD25- de bazo. Esta población está muy elevada en las zonas de implantación del tumor, sin embargo, para los experimentos de inhibición de T efectoras, se emplearon células de esta población de bazo, desconociendo la actividad de las T reguladoras (Tregs) locales.

En el modelo de colitis producido por la transferencia de células T CD4+ CD45RB^{high} vía intravenosa a la línea de ratón RAG-/-, las células transferidas procedentes del ratón CD69KO2 provocaron una mayor severidad de la patología frente a las del ratón WT. Este resultado puede ser justificado, ya que el ratón CD69KO2 presenta menor cantidad de Tregs (CD4+ FoxP3+) en los LNs mesentéricos y en la lámina propia en condiciones basales; y tras estimulación con péptido de OVA, el ratón OT-II CD69KO2 presenta también una disminución en las Tregs de bazo (106).

Otros trabajos establecen la expresión de CD69 en poblaciones Treg en LNs que drenan el tumor de pacientes con cáncer de cuello uterino (107), en sangre de individuos que rechazan en menor medida los trasplantes (108), y en estudios *in vitro* utilizando sobrenadantes de cultivos tumorales (109).

A la vista de todos los resultados, parece que CD69 es una molécula con funciones ambivalentes: puede ser estimuladora de la respuesta inmune o bien, estar presente en poblaciones que moderen dicha respuesta. Para aclarar este punto, es necesario conocer las investigaciones sobre CD69 en otro aspecto del sisteman inmune, su papel en la migración celular.

b) REGULADOR DE LA MIGRACIÓN DE LEUCOCITOS

El ratón *knock-out* de CD69 (CD69KO1 ni CD69KO2) no presenta diferencias en el porcentaje de las diferentes poblaciones hematopoyéticas localizadas en los distintos tejidos linfoides. Sin embargo, se generaron otras líneas de ratón transgénicas de CD69 que presentaban ligeras alteraciones en algunas poblaciones. En la línea transgénica en la que se eliminó el dominio citoplásmico de CD69 en el transgén (expresando su domino extracelular anclado a la membrana), las poblaciones tímicas estaban alteradas, presentando una menor proporción de timocitos dobles-positivos (DP) y mayor de timocitos maduros CD4+ CD8- (CD4SP) o CD4- CD8+ (CD8SP) (110). En este mismo trabajo también se utilizó una línea que sobreexpresaba la proteína completa de CD69 en el que se observó el mismo efecto. Otro modelo de sobreexpresión de CD69 *in vivo* arrojó los mismos resultados: el desarrollo tímico era normal pero existía un exceso de células maduras con menos capacidad de salir a periferia, ya que la retención era tanto mayor en cuanto más sobreexpresión del transgén había (111).

Sin embargo, el primer estudio que trata directamente la relación de CD69 con la migración celular es el trabajo de Shiow y col. (112), donde se demuestra que la expresión CD69 inhibe la expresión del receptor S1P1, provocando la retención de los linfocitos en los órganos linfoides. Anteriormente, ya se había descrito el papel fundamental que jugaba S1P1 en la migración de linfocitos (113-115). Este receptor para esfingosina-1-fosfato (S1P, *sphingosine-1-phosphate*) se expresa en una gran variedad de poblaciones, en altas concentraciones en células endoteliales y plaquetas, siendo necesario para la maduración vascular (116). Utilizando líneas de ratón *knock-out* (KO) condicionales, se observa cómo se requiere la expresión de este receptor para la migración de linfocitos T desde el timo y los órganos linfoides secundarios a sangre y linfa (114, 115). En esa línea, un conocido agonista de S1P1, FTY720, ha demostrado ser eficaz en el tratamiento de diversas patologías (117, 118).

Además, Srinivasan y col. (102) demostraron la relación entre el entrecruzamiento de S1P1 con la inhibición de la expresión de CD69 a través del factor de transcripción inducible por hipoxia 1 alfa (HIF-1α, *Hypoxia-induced Factor 1*) (119). En un estudio relevante sobre la regulación del tráfico de células T en el timo, se observó una salida acelerada de los timocitos a linfa en el ratón *CD69KO2* (120), donde se propone como función de CD69 la de retener los timocitos maduros varias horas en el timo. En el trabajo más reciente sobre CD69 y la migración (121) se observó que CD69 es necesario para la migración de células T *helper* de memoria a la médula ósea.

Tabla IN.1. Principales patologías con expresión de CD69 alterada en leucocitos.

PATOLOGÍA	POBLACIÓN CON NIVELES DE CD69 ALTERADOS (> ó <)	REFERENCIAS
Asma	Eosinófilos de BAL de enfermos (>)	(75)
Pneumonía eosinofílica	Eosinófilos de BAL de enfermos pero no de sangre (>)	(122)
Lupus (modelo de ratón)	Linfocitos T CD4+ de tejidos linfoides e infiltrados de riñón y pulmón, pero no de sangre (>)	(123)
Psoriasis	Linfocitos T de la epidermis lesionada (crónicamente activados) (>) pero no de la dermis	(124)
Psoriasis (modelo en ratón *fsn/fsn*)	Linfocitos T CD8+ de pLN (>)	(125)
Alzheimer	Monocitos/macrófagos de sangre periférica (CD14+) (>)	(126)
Artritis reumatoide	Células T de líquido sinovial de pacientes	(127)
Atopia	Neutrófilos de sangre periférica (>)	(128)
Linfoma de células B no Hodgkin	Células B tumorales (>)	(129)
Carcinoma hepatocelular	Linfocitos CD4+ CD25- de sangre periférica en pacientes (>)	(130)
Celiaquía	Células mononucleares de sangre periférica estimuladas con α-gliadina (>)	(131)
Endometriosis	Células CD56+ del endometrio de pacientes (>)	(132)
Síndromes coronarios	Células CD3+ en tejido afectado	(133)
Rechazo de trasplante renal	Linfocitos T de sangre periférica de individuos con rechazo	(134)
Rechazo de trasplante cardiaco	Linfocitos T de sangre periférica de individuos con rechazo	(135)
Gastritis	Linfocitos T CD4+ de las mucosas gástricas	(136)
Esclerosis sistémica	Linfocitos T (>) y linfocitos T CD4+ CD25high CD127low (Tregs) de sangre venosa (<)	(137)

En resumen, estos indicios apuntan a que la expresión de CD69 produce retención de linfocitos en órganos linfoides, y podría ser que también favoreciera la entrada al foco inflamatorio. Sin embargo, queda por demostrar la relación directa entre estos fenómenos, y por explicar por qué se produce migración de linfocitos en las líneas CD69 -/-. Otro objetivo de esta tesis será intentar resolver estas cuestiones.

En conclusión, surge la cuestión si CD69 actúa como regulador de la respuesta inmune o bien, participa en la distribución de los leucocitos, o bien ambas cosas relacionadas entre sí o no. La importancia de esclarecer la(s) función(es) de la proteína CD69 reside en la alteración de su expresión en numerosas patologías (ver Tabla IN.1). En la mayoría de casos se trata de una mayor proporción de células CD69+ en poblaciones situadas en el foco de infección/inflamación, que pueden ser diana de tratamientos efectivos.

3. REGULACIÓN DE LA EXPRESIÓN DE CD69

CD69 es una molécula que se expresa rápidamente en membrana, siendo detectable dentro de la primera hora tras la estimulación con PMA en linfocitos T humanos (67) y alcanzando su máximo sobre las 12 horas (48). Cabría pensar entonces que la regulación de su expresión puede ocurrir a diferentes niveles: por regulación de la transcripción mediante elementos *cis* y *trans*, por mecanismos de estabilización/desestabilización de RNA o por interacción con otras proteínas durante el transporte a la membrana con una forma preformada en el citosol, entre otras.

3.1. MECANISMOS DE REGULACIÓN OBSERVADOS

a) Regulación génica

El gen CD69 se encuentra en el brazo corto del cromosoma 12 en humano y en el 6 en ratón, dentro del complejo NK, donde la mayoría de los genes codifican para receptores con dominios tipo lectina de clase C (*C-type lectin-like receptors*), expresados en células NK (138, 139) (Fig. IN.3). Está presente en todos los genomas de mamíferos secuenciados hasta ahora (Fuente: VISTA *Genome* Browser (43)), no encontrándose en otras clases de vertebrados, salvo en una excepción: recientemente se descubrió su presencia en el genoma de *Gallus gallus* (140).

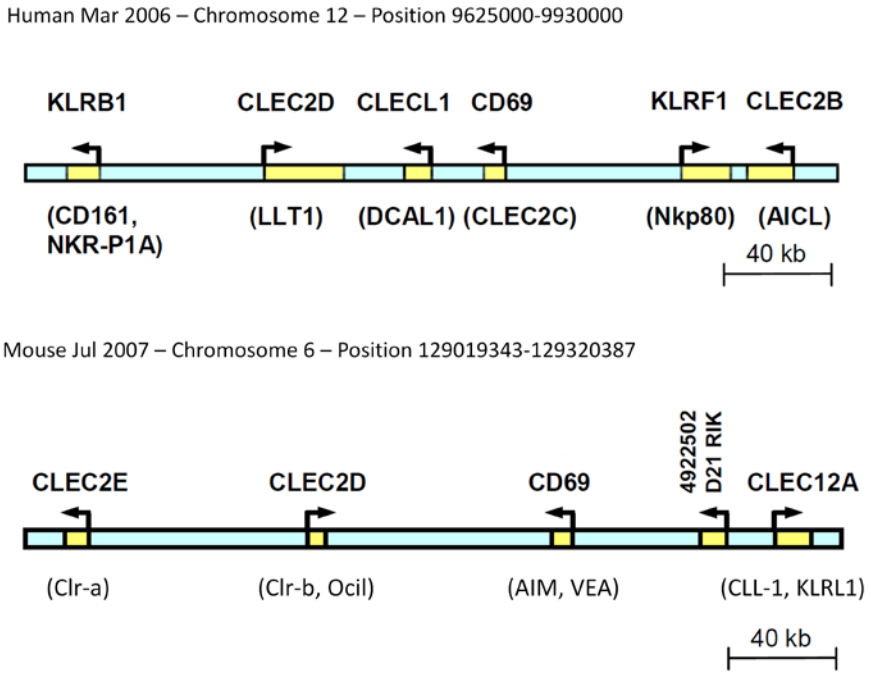

Fig. IN. 3. Posición de CD69 humano (fig. superior) y de ratón (fig. inferior) entre diferentes genes del complejo NK. Elaborado a partir de los datos obtenidos de *VISTA Genome Browser*: (http://pipeline.lbl.gov/cgi-bin/gateway2). El genoma base utilizado para la comparación está indicado en la parte superior de cada banda de genoma.

Consta de 5 exones, con un extremo 5' no traducido (5' UTR, *5' UnTranslated Region*) corto (<100 bp) y un 3' UTR largo (≈1000 bp). El promotor se caracterizó en ser humano (141) y en ratón (142). En el ser humano, la mínima unidad necesaria para la trascripción comprende desde las posiciones -78 a +16 y contiene las secuencias necesarias para la inducción por PMA, lo que se definió como promotor basal. La inducción en luciferasa se hace máxima cuando el fragmento llega hasta ~-700 bp, lo que se puede definir como promotor completo para ser humano. En ratón, Ziegler y col. definieron esta región como el fragmento desde -668 a +1.

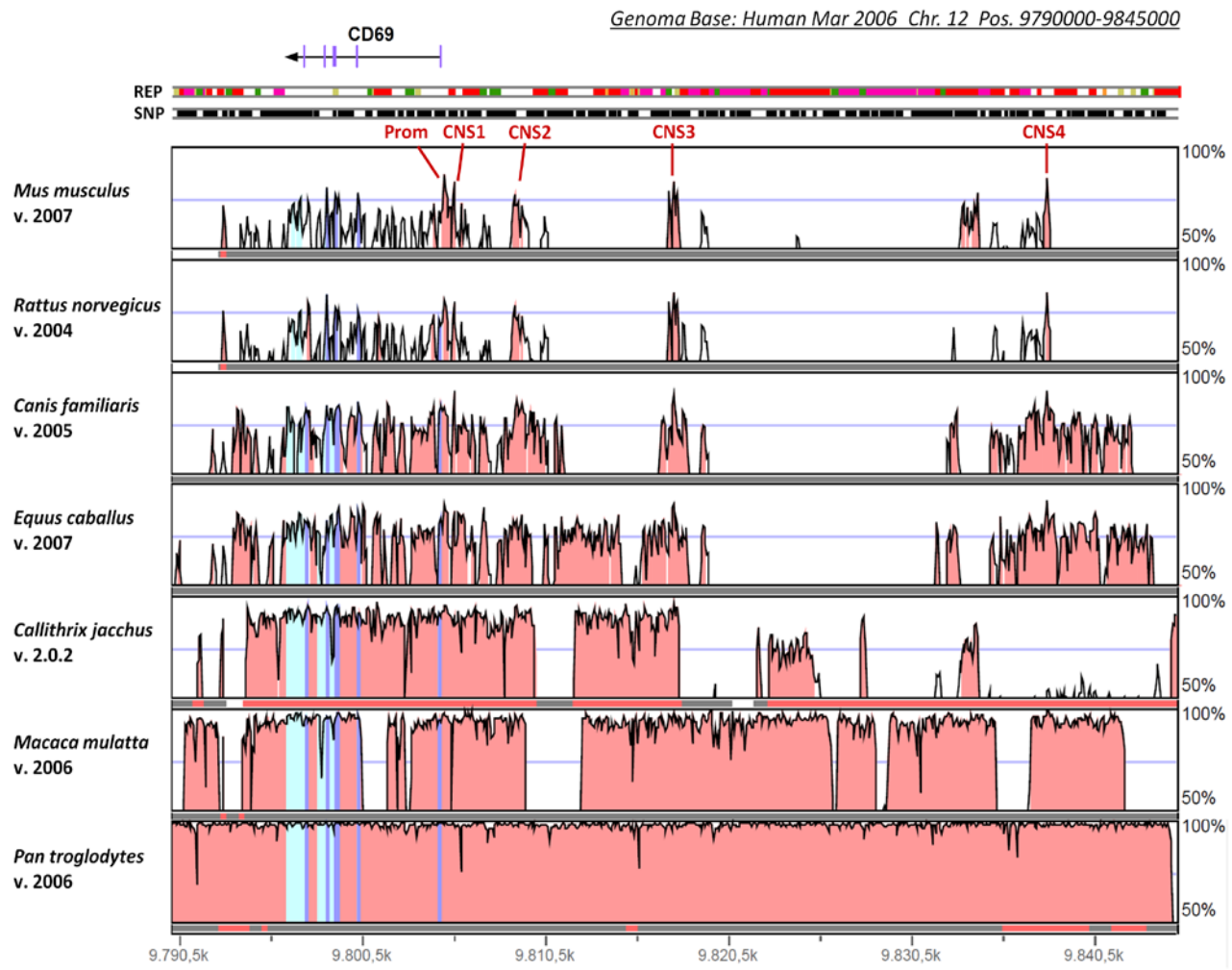

Fig. IN. 4. Conservación entre secuencias de distintos mamíferos en la zona del gen de CD69 y las regiones no codificante en 5'. Datos obtenidos de *VISTA Genome Browser*: (http://pipeline.lbl.gov/cgi-bin/gateway2). Las curvas indican la conservación enre la secuencia de ser humano (posiciones señaladas en la parte superior izquierda) y la de la especie indicada a la izquierda del gráfico. El grado de conservación mostrado varía desde un 50% hasta un 100% (parte derecha). En rojo están señaladas las zonas conservadas no codificantes; en morado, los exones; y en azul claro, las zonas transcritas no traducidas (*UTR*). Las barras en la parte superior indican repeticiones de secuencias (coloreada) y polimorfismos de un único nucleótido (*SNP*) (en negro). En la parte izquierda se hace referencia a la especie con la que se compara la secuencia de ser humano (secuencia indicada en la parte superior), y debajo del nombre científico figura la versión del genoma. En la parte superior se ha dibujado en gen de CD69 con sus exones en morado a modo indicativo.

Aparte del promotor, nuestro grupo ha definido recientemente 4 regiones reguladoras *cis* situadas en 5' respecto al gen de CD69, basado en su alta conservación entre las secuencias de ser humano y ratón (Fig. IN.4) (46). Estas regiones se denominan "secuencias conservadas no codificantes", CNS (*conserved non-coding sequence*), y han demostrado ser relevantes para la regulación de la expresión de genes relacionados con la respuesta inmune, como *IL-4* (143) e *Ifng* (45).

Las 4 regiones identificadas se denominaron CNS1, CNS2, CNS3 y CNS4, siendo la más próxima al promotor CNS1 y la más alejada CNS4 (Fig. IN.4). Todas ellas se encuentran en conformación "abierta" en el genoma de timocitos de ratón tanto con expresión constitutiva como sin expresión de CD69, analizado mediante ensayos de hipersensibilidad a DNasa I. Presentan marcas de activación transcripcional en histonas de manera dispar entre distintos tipos celulares, siendo CNS2 la más activa en timocitos, CNS1 en linfocitos T de LN, y CNS4 en células B de bazo (46).

La actividad luciferasa de los CNS en Jurkat demostró que CNS2 es el Amplificador (ver Sección 1 de Introducción) más potente en esta línea celular, y que potencia la inducción de la transcripción de CD69 por PMA. Sorprendentemente, *in vivo* en ratones transgénicos (TG), la construcción conjunta CNS2-CNS1-Promotor mostró una inhibición de la transcripción, empleando como reportero el cDNA de CD2 humano, además de una diferente regulación entre linfocitos T y B (46).

Por tanto, es necesario aportar más indicios sobre la función de CNS2 en la regulación de la transcripción de CD69; algunos de ellos se exponen en esta tesis.

Regulación mediante factores de transcripción

En el promotor de CD69 se han identificado una serie de elementos reguladores *cis* que se corresponden con sitios de unión a factores de transcripción (TFBS), y que regulan la actividad transcripcional del promotor. Es el caso de elementos de unión a RelA (miembro de la familia del factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas (NFκB, *nuclear factor kappa-light-chain-enhancer of activated B cells*)) (en linfocitos humanos), donde uno de ellos, κB2, localizado en -255 a -170, es necesario para la activación de la transcripción inducida por TNF-α (141).

En cambio, el factor de transcripción AP-1 (proteína activadora 1, *activator protein 1*) es necesario para activación por PMA, ya que la transfección de un dominante negativo de *c-fos* anula la inducción de la transcripción, mientras que IκB (inhibidor de NFκB (144)) sólo lo hace parcialmente (145).

Se desconoce el papel de estos elementos de unión a factores de transcripción en los 4 CNSs de CD69, resultando de especial relevancia su estudio en CNS2, que es otro de los objetivos de esta tesis.

b) Regulación de la disponibilidad del RNA mensajero

Tras activación con PMA y anti-CD3, timocitos y linfocitos T de LN en ratón (142), y con PMA en PBLs humanos (141); la activación de la transcripción parece instantánea, ya que se detectan tránscritos a los 30 y 60 minutos, respectivamente; y decayendo también en menos de 12 horas.

La rápida desaparición se puede explicar por la presencia de señales de degradación en el extremo 3' UTR, como se propone en el trabajo de Santis y col. (146), en el que un fragmento de la región 3' de CD69, rico en regiones AU, es suficiente para desestabilizar el gen de la β-globina respecto al 3' UTR de GM-CSF. Cabe destacar que el receptor se sigue expresando tras alcanzar el mínimo nivel de transcripción, indicando que existe otro tipo de regulación después de la traducción que mantiene a niveles bajos la expresión de la molécula.

Existen evidencias también de una regulación negativa de la expresión de CD69 por micro RNAs, tales como miR-130/301 (147) y miR-181 (148).

c) Regulación post-traduccional

La rápida cinética de expresión de CD69 puede indicar la existencia de péptidos en el interior celular en estado basal. Se han encontrado evidencias de la presencia de CD69 en el citoplasma de células T humanas de sangre periférica (68) y neutrófilos (70). En ambos casos la inducción de CD69 no necesita la nueva síntesis de RNA; incluso en el caso de los neutrófilos se ha descrito que CD69 intracelular se encuentra en un compartimento trans-Golgi, ya que la inducción no se ve afectada por cicloheximida ni por brefeldina A, aunque es cuestionado en otro estudio (72). Sin embargo, en el caso del linaje T, no se ha detectado la presencia de la proteína intracelular en las líneas Jurkat y CEM.

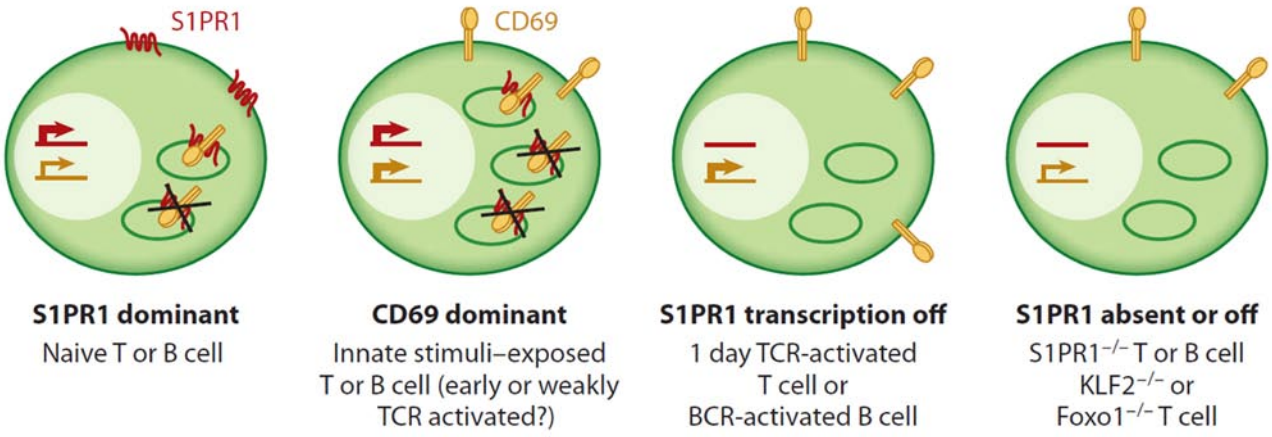

Fig. IN. 5. Modelo de regulación conjunta CD69-S1P1. *Izquierda*, cuando S1P1 (S1PR1) es abundante, por ejemplo en linfocitos *naive* y/o recién migrados a circulación, forma un complejo con CD69 y se internaliza, pudiendo volver a reciclarse o degradándose (*cruz negra*). *Centro izquierda*, recién activado el linfocito se induce rápidamente la expresión de CD69 y ocurre el proceso contrario. *Centro derecha*, tras 24 horas de la activación, la transcripción de S1P1 se inhibe y la expresión de CD69 es media-alta pero comienza a descender. *Derecha*, si S1P1 no se expresa por genotipo deficiente, la expresión de CD69 está aumentada.

Se ha propuesto recientemente una regulación conjunta de la pareja CD69-S1P1, en el que el exceso de expresión de uno inhibiría la expresión del otro mediante asociación por contacto e internalización, quedando el sobrante en la membrana (149, 150), ya que existen evidencias de interacción directa (151) y de inhibición de expresión recíproca (112) (Fig. IN.5)

3.2 VÍAS DE INDUCCIÓN DE LA EXPRESIÓN EN MEMBRANA

Se han encontrado diversas vías de activación de la expresión de CD69 en la membrana celular de los leucocitos. En linfocitos T, tanto el uso de PMA (152-155) como de anti-CD3 (156-158) o anti-CD2 (159-161) indican la activación de PKC previa a la expresión de CD69 (48, 162). Sin embargo, la inducción de la expresión a través de anti-CD28 (68),que actúa independientemente de PKC (163-165), sugiere la existencia de otras vías de activación de la molécula.

También se ha descrito la intervención de *p21ras* en la estimulación a través del TCR/CD3 (166) y de Raf1 en la activación por PMA (167), de linfocitos T; además de la activación por entrada de Calcio mediante el empleo de ionomicina (167).

Al igual que en células T; en linfocitos B (66) y células NK (168) se produce la inducción de CD69 tras estímulo a través de sus receptores de antígeno, seguramente a través de la inducción de PKC (169). También se ha observado para células de origen mieloide con otros ligandos fisiológicos (ver apartado 2.*1.* de Introducción).

En resumen, existen numerosas evidencias de que CD69 está regulado a múltiples niveles por diferentes vías de señalización, algunas de ellas específicas de linaje celular. Como objetivo de esta tesis se propone profundizar en la regulación de la expresión de CD69, a modo de comprender su funcionamiento.

OBJETIVOS

1. Profundizar en el conocimiento de la regulación transcripcional de CD69

   - Estudios *in silico* mediante búsquedas en bases de datos:
     - Identificación de posibles reguladoras de la transcripción de CD69 mediante el estudio de la conservación de secuencias de DNA entre diferentes especies animales.
     - Localización de sitios de unión de factores de transcripción a las secuencias de DNA de las posibles zonas reguladoras.
     - Accesibilidad de la cromatina en estas zonas reguladoras.

   - Estudios experimentales:
     - Accesibilidad del Intrón I de CD69.
     - Estudios funcionales en la zona reguladora CNS2.

2. Regulación de la expresión de CD69 en modelos de transgénesis de BACs conteniendo el gen de CD69

3. Estudios de los efectos de la sobreexpresión de CD69 *in vivo*.

CHAPTER I:

TRANSCRIPTIONAL REGULATION OF CD69 BY CONSERVED NON-CODING SEQUENCES

CAPÍTULO I:

REGULACIÓN TRANSCRIPCIONAL DE CD69 MEDIANTE ZONAS CONSERVADAS NO CODIFICANTES

INTRODUCTION

CD69 is an inducible receptor expressed in leukocytes. It is rapidly upregulated on the membrane of lymphocytes upon stimulation, as detected in T lymphocytes after 1 hour of treatment with PMA (67), while it reaches its maximum expression in myeloid populations in about 24 hours (73, 74, 77, 78). This specific regulation of CD69 expression observed in different subpopulations of leukocytes is suggested to be in part due to distinct transcriptional regulation mechanisms, as several *cis*-acting elements have previously been found in CD69 locus with lineage-specific effects on transcription (46).

On the human and mouse CD69 promoters, regulatory elements binding the Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NFκB), the Activator Protein 1 (AP-1), the Octamer family of proteins (Oct), the cAMP Response Element-Binding protein (CREB) and the Early Growth Response proteins (EGR) have been identified and proposed as responsible for inducible expression (141, 142, 145, 170). Other *cis*-regulatory regions have been identified previously by our group upstream of the CD69 gene (46). Those are four Conserved Non-coding Sequences (CNS): CNS1 is adjacent to the promoter, CNS2 is located at a distance of 9 kb in mice (4 kb in human), CNS3 at 29 kb (12 kb in human) and CNS4 at 40 kb (33 kb in human). They were defined based on their high homology (> 70% over 100 bp length) between human and mouse CD69 locus sequences, employing the *in-silico* approximation *Vista Genome Browser* (43) (see Section 3 of Introduction, Fig. IN-4). Those regions are expected to have critical roles, as orthologous sequences are significantly more similar than they would be expected to be if they were evolving under some reasonable model of neutral evolution. According to this, CNS have been found to be functional regulatory elements, also in immune-related genes, such as interleukin-4 (IL-4) (143), interferon gamma (IFN-γ) (45) or the Forkhead box P3 factor (FoxP3) (24).

Cis-acting regulatory elements usually present an open chromatin conformation, in a constitutive or inducible manner (10). Thus, DNase I hypersensitivity assays have been vastly used to detect them (12-15). We have previously shown that the four CNS are in open conformation in mouse thymocytes and possess marks of active transcription on histones. *In vitro* expression data functionally confirmed CNS2 as a potent transcriptional enhancer (46) (Fig I.0.1). In contrast, *in vivo* data employing a transgenic (TG) mouse line with the mouse CD69 promoter and different combinations of mouse CNS directing hCD2 expression,

showed an inhibition of transcription when CNS2 and CNS1 were upstream CD69 promoter. It also revealed a differential regulation between T and B cells.

A growing number of recent researches indicate that introns are also *cis*-acting elements and show important biological functions (171-177). Some studies have suggested that the first introns may play a vital role in transcriptional regulation of genes, acting as enhancers or repressors of transcription (178-183). Genomic first introns are enriched in TATA, CAAT and CG boxes, indicating putative regulatory roles

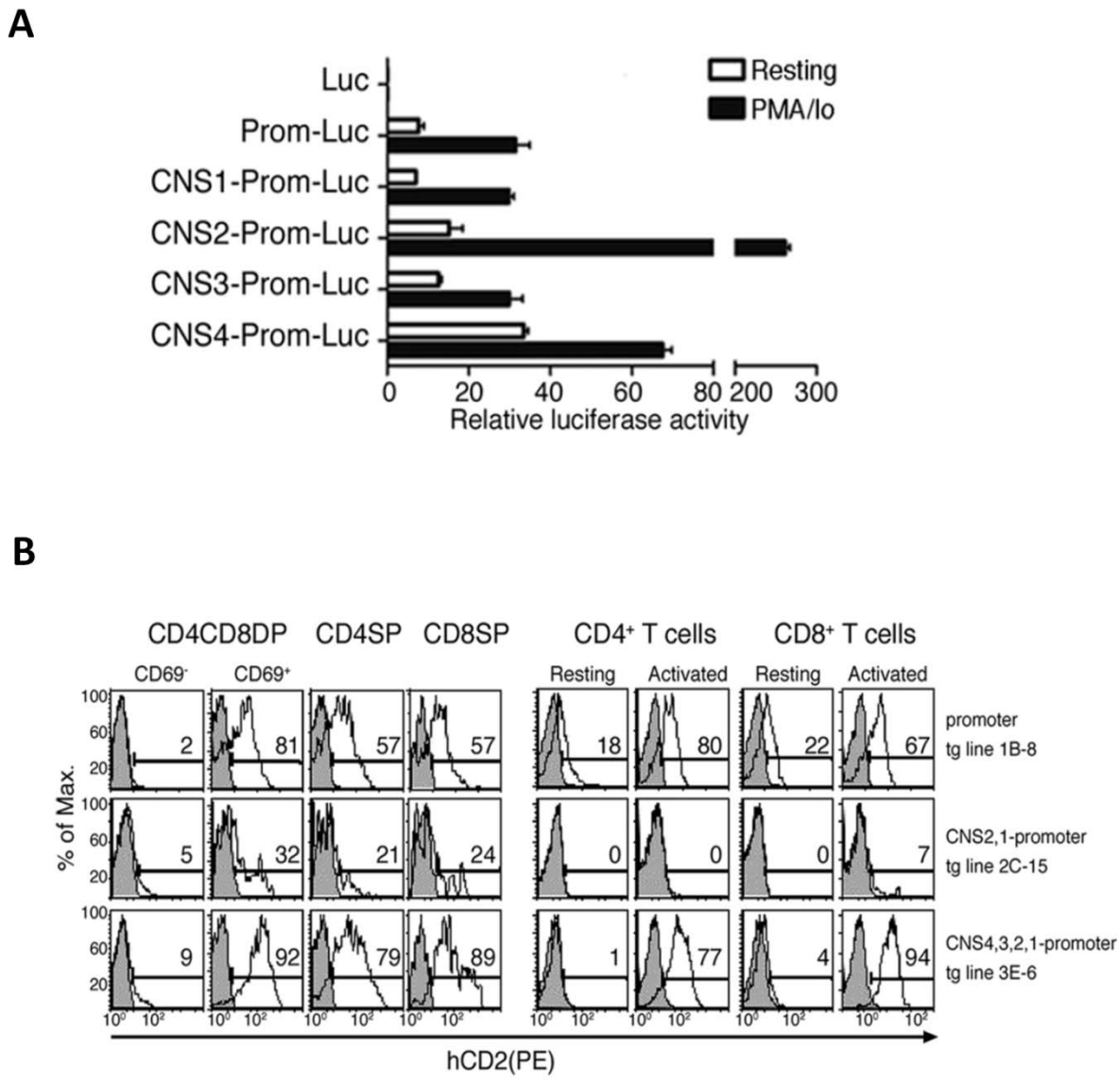

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Fig. I.0.1. Potential regulatory role of CNS2 in CD69 gene expression. A) mCNS2 enhancement activity on Prom-pXPG reporter vector. B) *In vivo* hCD2 transgenic expression directed by mCD69 promoter (*top*), CNS2 plus CNS1 plus promoter (*mid*), or CNS4 + CNS3 + CNS2 + CNS1 + promoter (*bottom*). Tissues analyzed include DP, CD4SP, CD8SP from thymus and CD4+, CD8+ from spleen.

(184). Several immune genes also have shown intron-mediated regulation, as an enhancer located in the second intron of the B-cell lymphoma 3 (BCL3) gene (185) or the presence of a second promoter in the intron of the recombination activating gene 2 (RAG2) (186). Consistently, some introns show hypersensitivity sites which function as *cis*-acting elements (187-189).

There are some evidences of transcription factors binding *cis*-regulatory elements that regulate transcription in a specific manner. For example, Notch signaling on the *Il4* enhancer CNS-2 has been detected to be determinant for expression in NK and memory CD4+ T cells (190). In the enhancer of the Granulocyte-macrophage colony-stimulating factor (GM-CSF), the transcription factors NFAT (Nuclear factor of activated T-cells) and GATA have been shown to regulate gene expression in a different way between myeloid and T cells (191).

RUNX (acronym of Runt-related transcription factor) is a family of transcription factors highly related to leukocyte development and lineage commitment, especially of T cells (reviewed in (192-194)). RUNX1 is required for normal hematopoiesis (195, 196) and for positive selection of thymocytes (197) (CD69 is expressed after this process (198). It also regulates T cell homeostasis and silences *Il4* promoter (199), being necessary the suppression of its activity by the T-helper-inducing POZ/Krüppel-like factor (ThPOK) to allow CD4+ T cell differentiation (200). Additionally, it is reported to form highly stable protein-DNA complexes in cooperation with E-twenty six (Ets) family of transcription factors, with remarkably frequent binding to T-cell specific enhancers (201-204).

Interactions of the E-twenty six-like factor 1 (Elk-1) with the Serum Response Factor (SRF) have also been described (205) forming a ternary nucleoprotein complex along with the Serum Response Element (SRE) located in the *c-fos* promoter (206). The ternary complex usually functions as a repressor, but can act as a transcriptional activator when it is phosphorilated by MAP kinases (207). GABPA or GABPα (GA-binding protein alpha chain) is the DNA-binding subunit of GA binding protein, which combines 2 DNA-binding domains: Ets-like (GABPA) and Notch-like (GABPB) (208). Due to its Ets-like domain, a redundant function with Elk-1 is proposed (209). However, GABPA is found in higher amounts than Elk-1's in Jurkat cells, but not in HeLa cell line (210). Moreover, it was proposed to function as an inductor in *Il-16* promoter (211) and *Il-2* enhancer (212).

Application of computer algorithms for the analysis and prediction of Transcription Factor Binding Sites (TFBS) is being developed and improved since early 80's (213), being considered as a reliable method for that purposes. In addition, data of sequencing of chromatin immunoprecipitation (ChIP-seq) from ENCODE consortium is available *in silico* to analyze in a variety of cell types (214).

In this chapter we analyze the role of Intron I and the four CNS upstream of CD69 gene previously defined as regulators of CD69 gene transcription by studying their conserved transcription factor binding sites. Additionally, we determine the functional relevance of the TFBS found in CNS2 measured by their transcriptional enhancer capacity. For that purposes, we employ *in silico* and functional procedures (*in vitro* luciferase assays, deletion of TFBS clusters, and site directed mutagenesis & silencing RNA of particular TFBS).

MATERIALS & METHODS

IN-SILICO GENOME WIDE REGULATORY INFORMATION

Comparative plots of conservation

For comparative genomic analysis of Intron I of CD69, DNA sequences of human and other different species were aligned by means of VISTA-browser (http://pipeline.lbl.gov/cgi-bin/gateway2) (43, 44). Different versions of human genome were employed: Human Mar 2006. Chr. 12, Pos. 9,796,000-9,805,200 & Human May 2009, Chr. 12, Pos. 9,904,600-9,914,300 for Fig. I.R.1.1; Human 2004, Chr. 12, Pos. 9,795,700-9,805,500 for Fig. 1.R.1.4; Human Mar 2006. Chr. 12, Pos. 9,804,764-9,805,880 (CNS1 plus promoter), Human Mar 2006. Chr. 12, Pos. 9,808,650-9,809,202 (CNS2), Human Mar 2006. Chr. 12, Pos. 9,817,153-9,817,696 (CNS3) & Human Mar 2006. Chr. 12, Pos. 9,837,823-9,837,990 (CNS4) for Fig. 1.R.2.1A, B, C & D respectively. Versions of genome of other species compared with human sequences are indicated in each figure.

Predicted conserved TFBS in Intron I & upstream CNSs of CD69

Sequences of the 4 CNS for the species human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), rhesus (*Macaca mulatta*), dog (*Canis familiaris*) and horse (*Equus caballus*), were downloaded from the online platform *Vista-Point* (http://pipeline.lbl.gov/cgi-bin/gateway2) from the portal *VISTA tools from comparative genomics* (http://genome.lbl.gov/vista/index.shtml) using as base genome the human genome version March 2006 from UCSC. For that purpose, we searched for the human sequences for each conserved fragment used previously for comparative analysis (indicated above) After that, we opened the *VISTA-Point* tool using the link "*alignment details*" and the corresponding orthologue sequence for each human CNS was shown with the associated link to download the data.

Secondly, we proceeded to introduce the intron I or CNS sequences into the application *Genomatix DiAlign* on the *Genomatix* website (http://www.genomatix.de/). The output data was obtained as an alignment with the conserved TFBS depicted in different colors reflecting their transcription factor families

(see Appendix I). To have a more graphical output of the data, a mammal (12 species) conserved plot comparing to each human regulatory region sequence was obtained from *University of California Santa Cruz (UCSC) Genomic Browser* (http://genome.ucsc.edu/cgi-bin/hgGateway). Finally, checking the positions of TFBS with the data from *Genomatix*, we marked them with arrows (orange arrows for TFBS conserved in the 6 species, green arrows, conserved in 5, or blue for conserved TFBS in 4 species).

Data from ENCODE consortium

Human open chromatin regions, histone marks and transcription factor binding by ChIP-seq in different cell lines are available from the ENCODE Consortium (214) (http://encodeproject.org/ENCODE/). All data were displayed on the University of California Santa Cruz Genome Browser. Input sequences employed were: Human 2006 chr12: 9,796,000-9,805,200 (Fig. I.R.1.3 & Fig. I.R.1.5); Human 2009 chr12: 9.912.000-9.920.000 (Fig. I.R.2.2); Human 2009 chr12: 9.922.000-9.953.000 (Fig. I.R.2.3).

ACCESSIBILITY ASSAYS

DNase I hypersensitivity assay

Human peripheral blood lymphocytes were used for the experiments. Red blood cells were lysed with lysis buffer (0.15 M NH4Cl, 1mM KHCO3, 1mM Na2-EDTA, pH 7.4) for 3 min at 4 ºC, subsequently washed in 30 ml of cold PBS and resuspended at a concentration of 2 x 10^6 per ml in complete media. Before DNase I digestion, human lymphocytes were stimulated *in vitro* with 10 ng/ml PMA (Sigma-Aldrich, St Louis, MO, USA) for 24 h.

After this time, cells (10^7 per ml) were permeabilized with 0.067 mg/ml lysolecithicin (Sigma-Aldrich) in buffer C (0.15 M Sucrose, 80mM KCl, 30mM Hepes pH 7.4, 5mM MgCl2, 5mM CaCl2) for 5 min and were left untreated or were treated with DNase I (Ribonuclease & Protease Free, Worthington Biochemical Co, NJ, USA) for 10 min on ice. DNase I was added at a final concentration of 0, 5, 10, 15, 25, 35 and 45 U/ml. Reactions were stopped by the addition of EDTA, SDS and Proteinase K to final concentrations of 10mM, 0.4% (w/vol) and 0.4mg/ml, respectively, and were incubated overnight at 37 ºC. DNA was purified by phenol, phenol-chloroform and chloroform extractions and ethanol precipitation. For southern

blot, genomic DNA was re-suspended in conventional TE buffer (10mM Tris-HCl pH 8, 1mM EDTA) and for quantitative PCR analysis in TE_{10} buffer (10mM Tris-HCl pH 8, 0.1mM EDTA).

Measurement of chromatin accessibility by southern blot

Purified DNA (10 μg) was incubated overnight at 37 ºC with an excess of NdeI restriction enzyme and was separated by 0.7% (w/vol) agarose gel electrophoresis. DNA was denatured by soaking the gel in 0.5 M NaOH/1.5 M NaCl for 45 min and neutralizing it in 1 M Tris-HCl pH 8, 1.5M NaCl for 45 min. DNA was transferred to nitrocellulose membranes by capillary action in 20X SSC (3 M sodium chloride and 300 mM trisodium citrate, adjusted to pH 7.0 with HCl) overnight, followed by cross-linking with UV light (Stratalinker, Stratagene, La Jolla, CA, USA). Blots were prehybridized for 3 h at 42 ºC with hybridization solution (50% formamide, 5X Denhardt's solution, 0.5% (w/vol) SDS, 50mM Hepes pH 7) containing 100 mg/ml denatured salmon sperm DNA and were probed with denatured ^{32}P-labeled DNA fragments (Invitrogen Random primer kit, Invitrogen, Carlsbad, CA, USA). Primers used to generate probe S1-H were S1 (F): 5'-CCCTCTGTACAATGGTGAAACA-3' and S2 (R): 5'-GCAACTTCTCTTGGTCCAGTTT-3'. Blots were washed twice in 1X SSC, 0.5% (w/vol) SDS for 15 min at room temperature, twice in 0.1X SSC, 0.1% (w/vol) SDS for 15 min at 60 ºC and once in 0.1X SSC, 0.1% (w/vol) SDS, 0.1 mg/ml proteinase K for 30 min at 37 ºC. DNA bands were visualized by autoradiography.

LUCIFERASE ASSAYS

Plasmids

Mouse CD69 promoter (-1 to -609, BAC clone RP24-188C4) was cloned into BglII and HindIII RE cloning sites of the commercial luciferase vector pGL3 basic (Promega) (*pPr*). After that, *pPr2* vector was generated by cloning mouse CNS2 region (chromosome 6: 129234359-129235318, Mouse Genome version 2010, UCSC Genome Bioinformatics (215)) into KpnI and XhoI RE sites, introducing an EcoRI site by KpnI for further cloning.

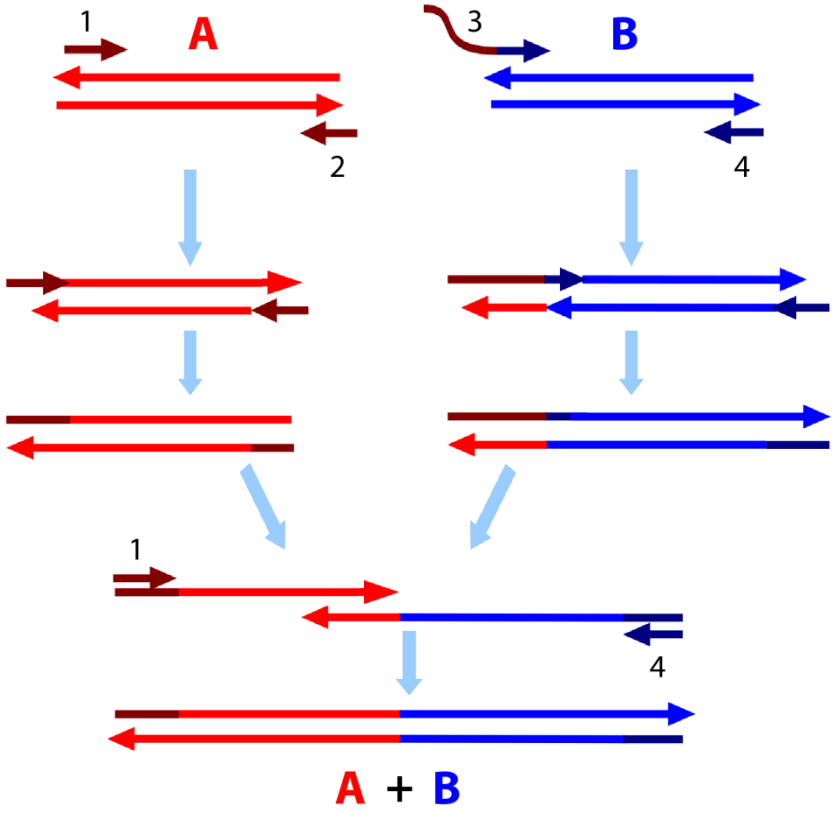


Fig. I.M.1. Overlap extension PCR. DNA fragments from different sources can be joined by designing primers overlapping their junction. Only 20-nt of sequence overlap of each pair of joining-fragments in one primer is required.

DNA inserts of *pPr2ΔA, pPr2ΔB, pPr2ΔC, pPr2ΔD* were cloned into EcoRI and XhoI sites of *pPr* plasmid. To generate the complete sequences for insertion, an overlap PCR (216) was employed using primers shown in Table I.M.I. Overlap PCR consists in fusing 2 or more fragments using primers which overlap those fragments (Fig I.M.1). To join 2 fragments, only one primer overlapping 20 nt with the other fragment is required. After that, a new PCR is settled with both fragments as DNA templates and only the pair of primers hybridizing at the ends of the complete fragment. This technique is further explained in Section II.M from Chapter II.

Site-Directed Mutagenesis

Site-directed mutagenesis allows mutating selected single nucleotides on a sequence of DNA (217-219). A high efficient and simple method has been developed to point-mutate base pairs in plasmids: the

Quikchange method (38). It consists in amplifying a plasmid with a pair of complementary primers mutated in their central part respect to original vector. The amplified circular plasmids contain the mutated sites and the parental vector is digested with *DpnI*, a restriction enzyme specific for methylated DNA (parental plasmid from *E. coli*). Then, the non-methylated newly-synthesized mutated copies of plasmid are transformed into competent cells. Typical efficiency is > 80 % (Fig. I.M.2).

The kit *QuikChange Lightning Site-Directed Mutagenesis kit* (Agilent) was employed in this assay following manufacturer instructions. Every PCR product and *DpnI* digestion was checked by agarose gel electrophoresis previous to transformation in bacteria.

Luciferase assays

The technique is based on directing the firefly luciferase gene expression by promoters/enhancers or other regulatory elements. It is necessary then to clone those DNA fragments in the plasmid containing the

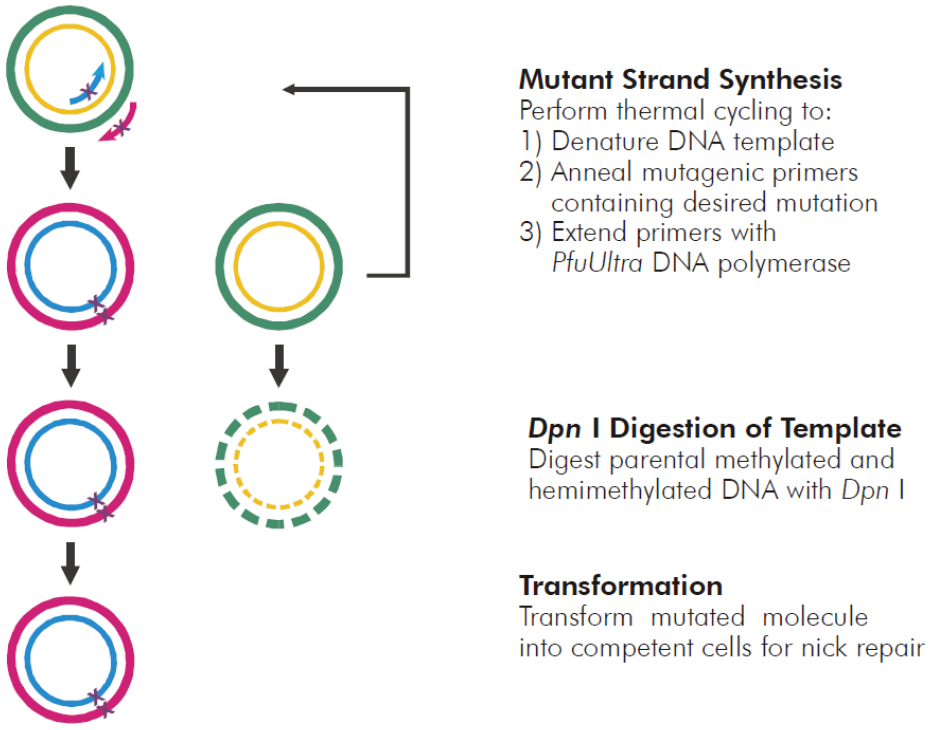


Fig. I.M.2. Overview of the QuikChange II site-directed mutagenesis method. Agilent Technologies, Stratagene Products Division.

luciferase cDNA. To standardize the luciferase activity independently of the efficiency of transfection between samples, a vector containing *Renilla* luciferase is co-transfected at low doses to estimate proportions between firefly product (oxyluciferin) and *Renilla* product (coelenteramide). A commercial kit is specially developed to read consecutively both luciferase signals, using 2 reagents: first, *LARII* catalyzes firefly reaction and second, *Stop & Glo* quenches the first reaction and starts *Renilla* activity (*Dual Luciferase Kit* from Promega).

Table I.M.I. Primers employed for Luciferase Assays.

NAME	5' -> 3' SEQUENCE
pPr_1F	GAGATCTGTCCAATTGAGAGAGAGGGAGA
pPr_2R	CAAGCTTCCTTTTTAATTTTTTTCCCC
pPr2_1F	CGGTACCAGTGAATTCTGCTGCATGTCTTCCTCTCA
pPr2_2R	CCTCGAGTCCACCTGCTTCTGCCTATT
pPr2.1_2F	CCATTCAGACACTAAAACCCACTGAAAGGAAATGATGTAAT
pPr2.1_1R	TGGGTTTTAGTGTCTGAATGG
pPr2.2_2F	AGCTGCCGTGATAAGGACTGTA
pPr2.2_1R	TACAGTCCTTATCACGGCAGCTCTTTCAGTGAGTGAATGAG
pPr2.3_2F	CCAGTACCTCATTCACTCACTG
pPr2.3_1R	CAGTGAGTGAATGAGGTACTGGACATGGGACCAGTAAAGGT
pPr2.4_2F	ACTGGTCCCATGTAGTAACC
pPr2.4_1R	GGTTACTACATGGGACCAGTGGTAAAATTGTGAAGTTCCTG
pPr2.R_F	GCCTCAGGTCGGAAGTTTGTTTTTCCTTTGAACACATACCAC
pPr2.R_R	GTGGTATGTGTTCAAAGGAAAAACAAACTTCCGACCTGAGGC
pPr2.G_F	CCGGAAACTGCCTCAGGTCTTAAGTTTGTGGTTCCTTTGA
pPr2.G_R	TCAAAGGAACCACAAACTTAAGACCTGAGGCAGTTTCCGG
pPr2.GE_F	GTTGTCACCGGAAACTGCCTCACCTCGGAAGTTTGTG
pPr2.GE_R	CACAAACTTCCGAGGTGAGGCAGTTTCCGGTGACAAC
pPr2.S_F	AACCTCCCTCCGGATTCTTGGTATAAGGTCACACTTGAGG
pPr2.S_R	CCTCAAGTGTGACCTTATACCAAGAATCCGGAGGGAGGTT
pPr2.C1_F	TCCTTTCAGAAACCTCCCTCCAAATTCTTCATATAAGGTCACAC
pPr2.C1_R	GTGTGACCTTATATGAAGAATTTGGAGGGAGGTTTCTGAAAGGA
pPr2.SR_F	pPr2.R_F in *pPr2.S* plasmid
pPr2.SR_R	pPr2.R_R in *pPr2.S* plasmid
pPr2.SRG_F	ACCGGAAACTGCCTCAGGTCTTAAGTTTGTTTTTCCTTTGAAC in *pPr2.SR*
pPr2.SRG_R	GTTCAAAGGAAAAACAAACTTAAGACCTGAGGCAGTTTCCGGT in *pPr2.SR*

Jurkat T cells (5-7 x 10^5), K562, U937 (2-3 x 10^5) were transfected with 1 μg of pGL3b and pGL3Pb derivatives (purified with *Plasmid Maxi Kit* from Qiagen) plus 20 ng of pRL-TK (Renilla luciferase plasmid from Promega) using *Superfect* (Qiagen) following manufacturer's protocol. Cells were cultured at 37 ºC with 5% CO_2 for 24 h. Then, cells were stimulated or not with 10 ng/ml of PMA and 500 ng/ml of Ionomicin, PMA alone or 5 μg/ml plate-bound anti-CD3 (clone OKT3; eBioscience) and anti-CD28 (clone CD28.2; eBioscience) mouse antibodies or were mock incubated, for other 24 hours. 48 h after transfection, cells were lysed using *Passive Lysis Buffer* (Promega) and luciferase activity (firefly/renilla) was measured with a dual luciferase kit (Promega).

SILENCING OF hRUNX1 BY NUCLEOFECTION

Nucleofection

RNA silencing was produced using *Cell Line Nucleofector® Kit V* from Amaxa and siRNAs *siRUNX1-59* (ref: s2459) and *siNeg* were from Ambion. 10^6 Jurkat cells per point were washed 3 times in 1x PBS and resuspended in 100 μl of *Cell Line Nucleofector Solution V*. Then 600 ng of *siRUNX1* or *siNeg* were mixed with the cell suspension in an Amaxa certified cuvette and nucleofected applying the program X-05 in the Amaxa Nucleofector. After waiting for 10 min at room temperature, cells were harvested with 500 μl of pre-warmed complete medium rinsing the cuvette, transferred to a 6-well culture dish and incubated at 37 ºC and 5% CO_2 for 24 h in a final volume of 1 ml of complete medium. After that, cells were harvested or stimulated with 10 ng/ml PMA plus 500 ng/ml Ionomicin for 24 extra hours.

RNA extraction

RNA extraction was carried out employing *NucleoSpin® RNA/Protein* kit from Macherey-Nagel (REF: 740933). Cells nucleofected for 24 hours (unstimulated) or nucleofected for 24 hours and stimulated for other 24 h were washed in cold 1x PBS and resuspended in 350 μl of buffer RP1. Lysates were filtrated with *NucleoSpin Filter units* and the filtrates were mixed with 350 μl of cold 70% Ethanol. The whole mix was added to *NucleoSpin RNA/Protein columns* to bind RNA. Columns were desalted with buffer MDB and the DNA was digested with DNase for 15 min following manufacturer directions. Then, columns were washed with RP2 and RP3 buffers and RNA was eluted in 60 μl of RNase-free water.

Table I.M.II. Touch-down protocol program for Real- Time PCR.

Program (Analysis Mode)	Cycles	Segment	Target Temperature	Hold Time	Acquisition Mode
PreIncubation (None)	1		95 ºC	10 min	None
Amplification (Quantification)	2	Denaturation	95 ºC	10 sec	None
		Annealing	65 ºC		None
		Extension	72 ºC		None
Amplification (Quantification)	2	Denaturation	95 ºC	10 sec	None
		Annealing	64 ºC		None
		Extension	72 ºC		None
Amplification (Quantification)	2	Denaturation	95 ºC	10 sec	None
		Annealing	63 ºC		None
		Extension	72 ºC		None
Amplification (Quantification)	2	Denaturation	95 ºC	10 sec	None
		Annealing	62 ºC		None
		Extension	72 ºC		None
Amplification (Quantification)	2	Denaturation	95 ºC	10 sec	None
		Annealing	61 ºC		None
		Extension	72 ºC		None
Amplification (Quantification)	35	Denaturation	95 ºC	10 sec	None
		Annealing	60 ºC		None
		Extension	72 ºC		Single
Melting Curve (Melting Curves)	1	Denaturation	95 ºC	0 sec	None
		Annealing	60 ºC	60 sec	None
		Melting	95 ºC		Continuous
			Ramp Rate = 0.1 ºC/sec		
Cooling (None)	1		40 ºC	30 sec	None

Reverse transcription

cDNA was synthesized using AMV Reverse Transcriptase from Promega (REF: M510). For priming the reaction, 1 µg of total RNA was mixed with 0.5 µg of random primers or oligo dT primers and then heated to 70 ºC for 5 min and chilled on ice for 5 more min. To set the reverse transcription reaction, the following reagents were added: 5 µl of *AMV Reverse Transcriptase 5X Reaction Buffer*, 2.5 µl of dNTPs 10 mM, 1 µl of AMV Reverse Transcriptase and Nuclease-free Water to a final volume of 25 µl. The mix was incubated at 42 ºC for 1 hour and then frozen at -20 ºC overnight.

Real-time PCR

Real-time was performed using *LightCycler® FastStart DNA Master^{PLUS} SYBR Green I* from Roche (REF: 03515885001). First, cDNA samples were diluted 25 times with PCR-grade water to avoid reverse

transcriptase buffer interference in the PCR. Standard curve samples were obtained mixing 1:1 all the samples and diluting the mix 10, 25, 50 and 100 times with PCR-grade water.

Relative quantification was carried out amplifying hCD69 and RNA 18s (housekeeping RNA). Primers for hCD69 amplify a 50nt-amplicon located between exons 1 and 2. The primers used were:

hCD69_F: 5'-CAGTCCAACCCAGTGTTCCT-3'

hCD69_R: 5'-CGTGTTGAGAAATGGGGACT-3'

RNA18S_F: 5'-CTCAACACGGGAAACCTCAC-3'

RNA18S_R: 5'-CGCTCCACCAACTAAGAACG-3'

The protocol employed was a touch-down protocol (220) (see Table I.M.II) to avoid unspecific amplification of impurities.

Flow cytometry of human cell lines

Human cells were immunostained for analysis by Flow Cytometry. Staining was done for 20 min at 4º C with PE-Cy7- conjugated anti-human CD69 antibody diluted in staining buffer (1x PBS supplemented with 2% of Fetal Bovine Serum and 2mM of EDTA) Cells were washed twice with staining buffer and 7-AAD (BD Pharmingen) was added after washing to exclude dead cells.

Samples were analyzed with flow cytometer *FACSCanto* (Becton Dickinson) and the software employed to analyze the data was *FACSDiva* (Becton Dickinson).

RESULTS

1. REGULATORY PROPERTIES OF CONSERVED NON-CODING SEQUENCES IN CD69 GENE

As large first introns have been revealed to contain important *cis*-acting elements (see Introduction), we intended to study intron I of CD69, which spans 4345 bp in human and 3765 bp in mice, for CNSs. Our group previously described four upstream conserved non-coding sequences (CNSs) for CD69 identified by comparison among different mammal species sequences, and analyzed for their regulatory properties (46).

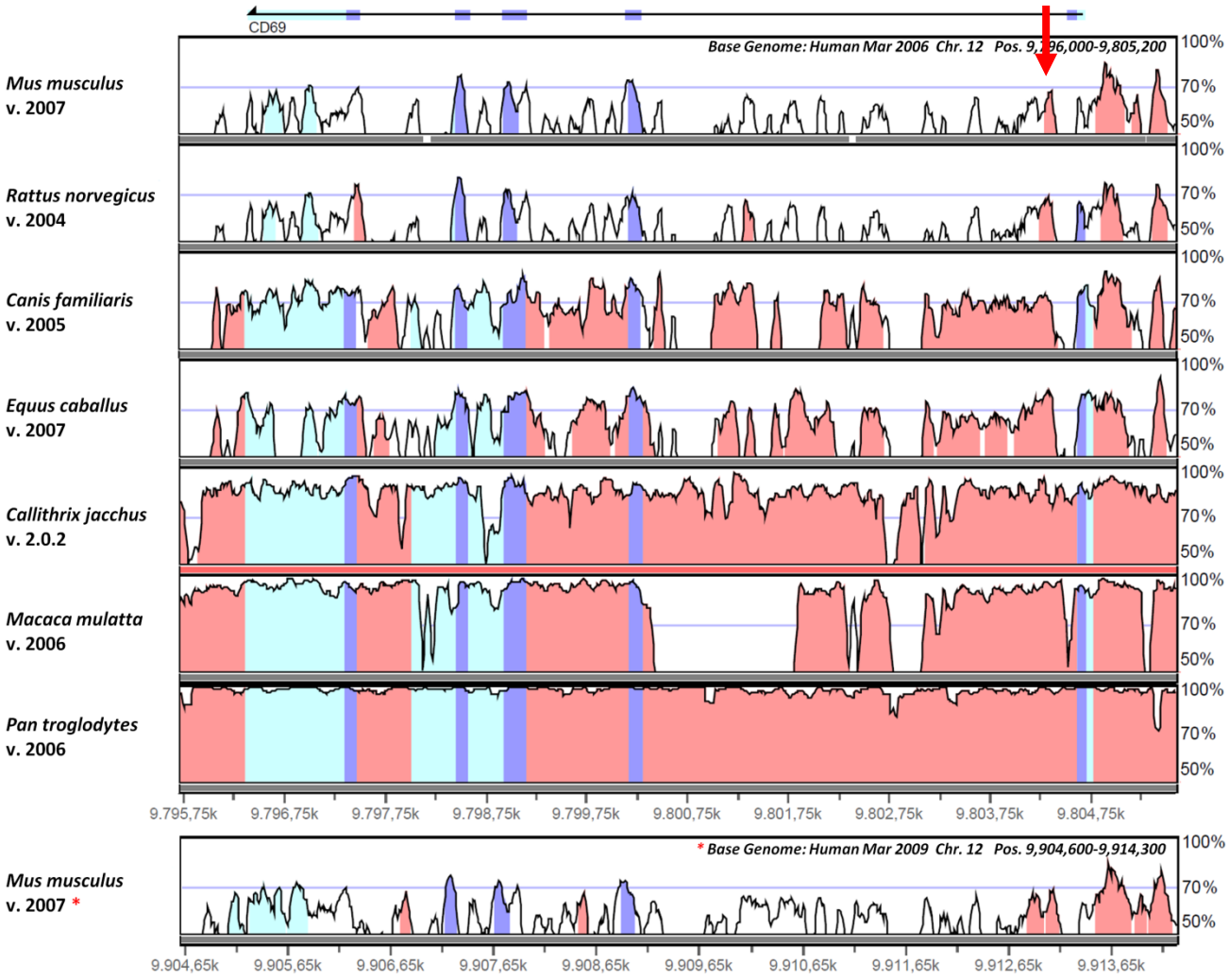


Fig. I.R.1.1. Conserved non-coding sequences (CNSs) within CD69 gene. VISTA tracks (http://pipeline.lbl.gov/cgi-bin/gateway2) where paired sequence conservation between human CD69 gene and organism referred in *left* for each case are plotted. Color red indicates CNSs; purple, exons; and light blue, untranslated regions. *Bottom*, same plot as the one on top, but using as genome reference human version 2009 instead of version 2006. Positions of human genome considered, are showed in top right of graphs. Red arrow points to intronic CNS, *iCNS*.

Using this approach, however, sequence analysis indicates that Intron I is less evolutionarily conserved (Fig. I.R.1.1). A small CNS is present proximal to Exon I in the human-mice comparison (iCNS), but no other region in Intron I fulfills the criteria to be considered a CNS (70% identity over 100 bp length). When human genome version of 2009 is employed as base genome, iCNS appears to be longer, but similarly no other CNSs in Intron I were obtained (Fig. I.R.1.1, bottom).

Chromatin accessibility of human CD69 intron I

As described for other tissue-specific enhancers (221), CD69 gene may contain an intronic DNA regulatory region that is not conserved in a canonical basis. To study that possibility, human peripheral blood lymphocytes (PBLs) were cultured with or without PMA for 24 hours and were subsequently treated with increasing amounts of DNase I. Genomic DNA was purified, digested with NdeI enzyme and analyzed by southern blot using probe S1.H (Fig. I.R.1.2a). This assay identified multiple bands in resting and activated peripheral blood lymphocytes (Fig. I.R.1.2b). Remarkably, a hypersensitivity site (HS) was clearly located in intron I (indicated as *HSa*) in unstimulated and stimulated cells, being more prominent after stimulation. Importantly, this is the first inducible HS described for the CD69 gene. It is possible that this region participates in the induction of CD69 expression through the recruitment of stimulation-dependent transcription factors (TFs). Pre-bound TFs in CD69+ cells to this intronic site may also exist, as the HS is

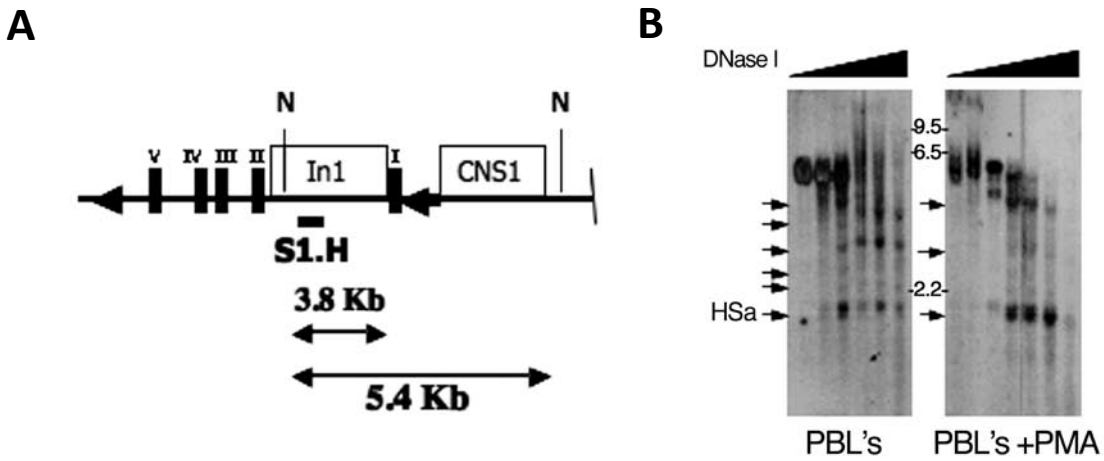


Fig. I.R.1.2. Chromatin accessibility of human CD69 intron I. A) Map of the human CD69 locus showing locations of significant features (In1: Intron 1; CNS1) and the genomic fragment expected from NdeI digestion. Probe used for Southern blot (S1.H) is also shown. B) DNase I analysis using S1.H probe. Human peripheral blood lymphocytes were activated with 10 ng/ml PMA for 24 h or were left untreated, and were subsequently treated with 0, 5, 10, 15, 25, 35 and 45 U/ml of DNase I. NdeI digested DNA was examined by Southern blot. Size markers and relevant bands (HS sites) are denoted.

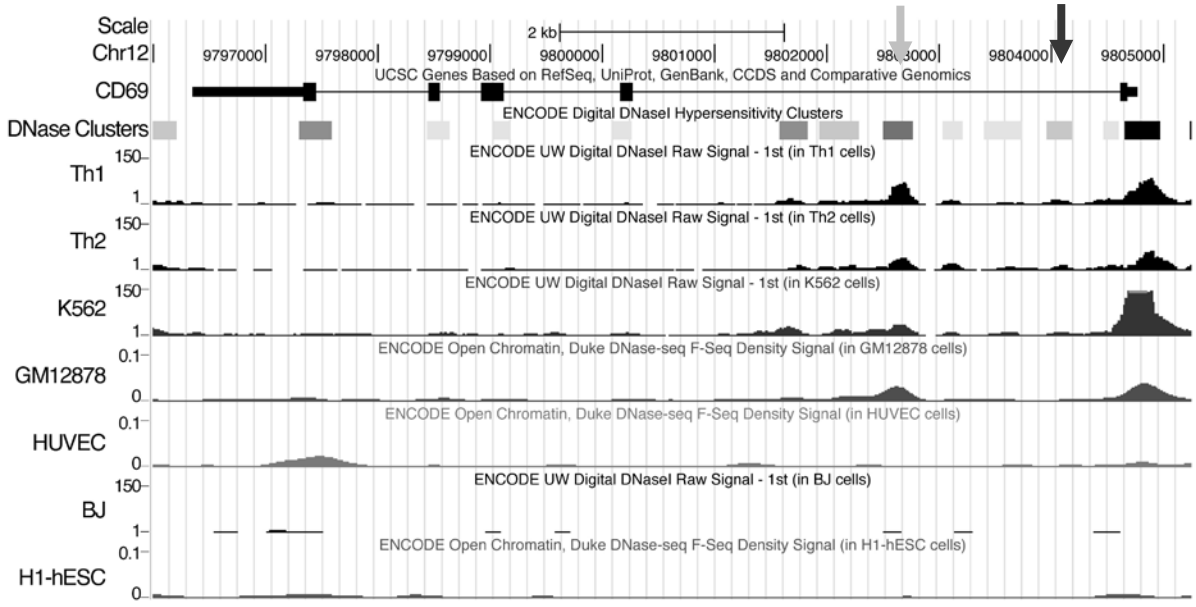


Fig. I.R.1.3. UCSC Genome Browser display on human CD69 locus. ENCODE DNase I hypersensitivity track from Duke and Washington University in Th1, Th2, K562, GM12878, HUVEC, BJ and H1-hESC cells. Red arrow points to HSa, and blue arrow, to iCNS.

also present in unstimulated cells.

Human peripheral blood lymphocytes contain a mixture of different immune cells such as T and B-lymphocytes and NK cells. As an attempt to identify whether this intronic HS was cell-type specific, we analyzed open chromatin regions using public data from the ENCODE consortium in the University of California Santa Cruz (UCSC) Genome Browser (Fig. I.R.1.3).

Two remarkable results were obtained from this analysis: first, a marked HS in intron I is observed in primary human Th1 and Th2 immune cells, in untreated GM12878 B cells and in erythroleukemic K562 cells, and, second, additional weaker HSs are also present in human Intron I concentrated 2 Kb downstream of the promoter (Figure I.R.1.3). This HS cluster is present in different human immune cells and, therefore, it is not cell-type specific. In addition, all HSs are absent in endothelial HUVEC cells, BJ fibroblasts and embryonic stem cells H1-hESC, indicating that this regulatory region is only active in cells that express CD69 or are competent to do it. The HS marked with a red arrow corresponds to the experimentally identified *HSa* (Fig. I.R.1.2b) as they both map to the centre of the intron I and they are the strongest in intensity. In contrast, iCNS is showed as a weak HS in hematopoietic cells and totally absent in other cell types (Figure I.R.1.3, blue arrow).

Common transcription factor-binding sites in CD69 intron I

Compared sequences for 6 mammal species (human, mouse, rat, rhesus, dog and horse) of the CD69 Intron I were analyzed to find conserved TFBS, employing the online-available software *Genomatix DiAlign*. Despite the absence of CNSs, several TFBS were found in the most conserved zones of the intron; however, only 2 are conserved in all species studied: interferon response factor 4 (IRF4) and BRNF (Fig. I.R.1.4). Regarding HSa, one of this highly conserved sites, IRF4 binding site, was located within it, in addition to SMARCA3 and Blimp (found in human but not in mice). Not surprisingly, HSa is located in a high conserved sequence among mammals (Fig. I.R.1.4, middle, labeled as "conservation"). Due to its short length, it is not considered a CNS by VISTA platform, but actually its TFBS are highly conserved (see raw data from *Genomatix* in Appendix I)

Apart from the highly confident statistical data obtained from in *silico* tools, ENCODE project contributes to regulatory studies with whole-genome experimental data, like ChIP-seq, which provides TFBS for different human cell lines (50). Therefore, we analyzed the available data from ChIP-seq of Intron I

The findings obtained by software prediction were in agreement with Chip-seq data from the ENCODE

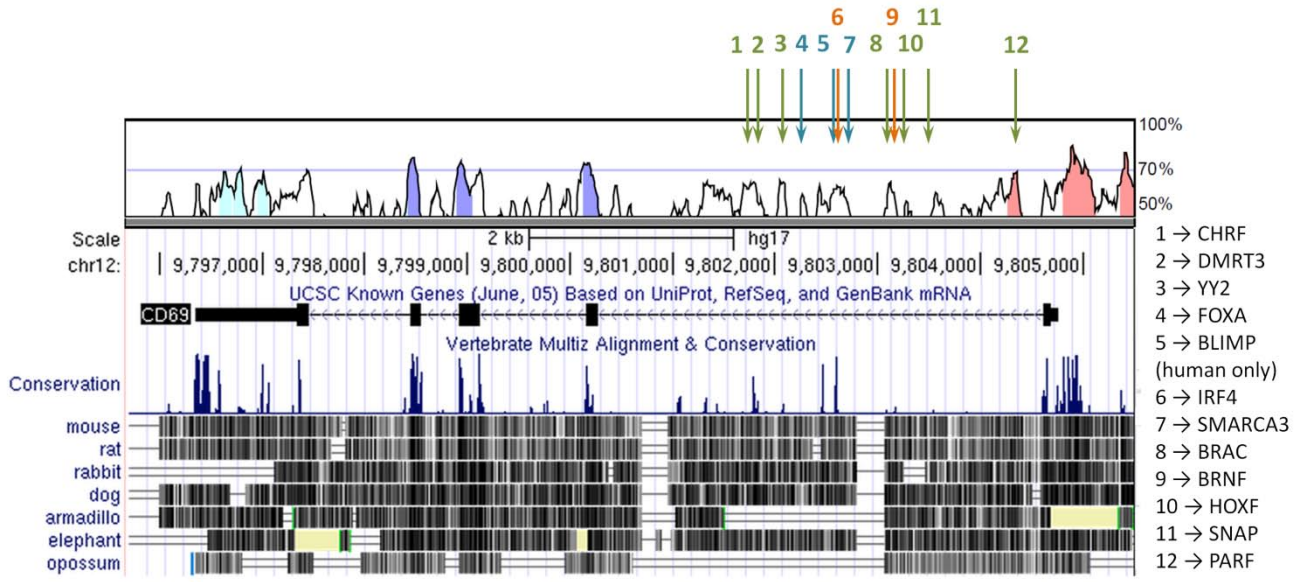


Fig. I.R.1.4. Conserved TFBS in Intron I of CD69. Data from TFBS conserved in 4 (blue arrows), 5 (green arrows) or 6 species (orange arrows) from a total of 6 mammal species (human, mouse, rat, dog, horse and rhesus) were obtained from *Genomatix DiAlign* (http://www.genomatix.de/) and plotted (*top*) over a VISTA conservation track between human and mice sequences for CD69 gene. Red zones, CNSs, in purple, exons, and in light blue, unstranslated regions. *Bottom*, track of conservation among mammal species indicated on *left*, obtained from *USCS Browser* (http://genome.ucsc.edu). Numbers on *right*, legend of TF that bind to sites indicated by arrows from TFBS data (*top*). Base sequence: human 2004 (UCSC). Position displayed: chr12: 9,795,700-9,805,500

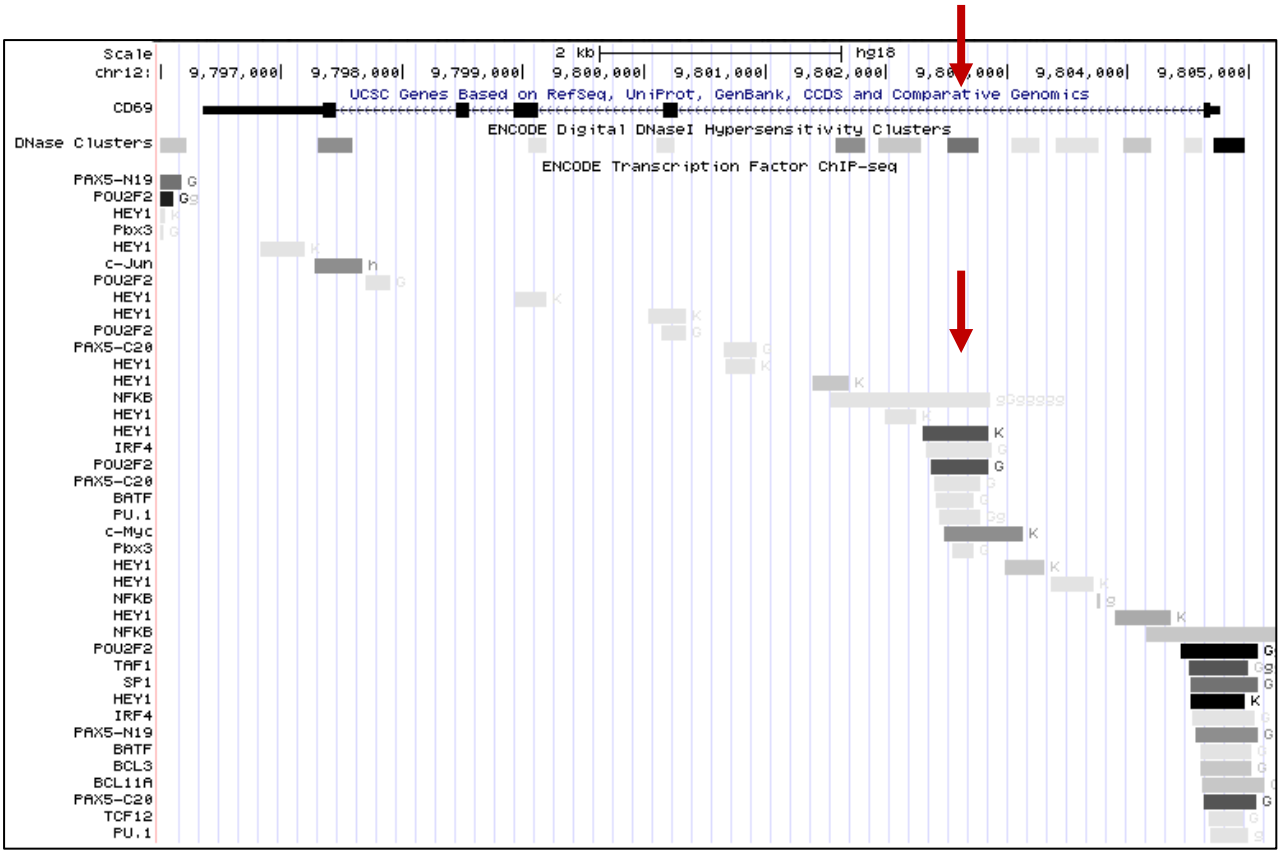


Fig. I.R.1.5. TFBSs from ChIP-seq displayed on human CD69 locus. Analysis of Chip-seq data from the ENCODE consortium. HSa (region chr12: 9802297-9802852), enriched in several transcription factors, is marked with red arrows. Capital letters next to bars indicates the cell line where the transcription factor is bound: K, K562 cell line; G or g, GM12878 cell line or similar. Base sequence: human chr12 2006 (UCSC). Position displayed: chr12: 9,796,000-9,805,200.

consortium in which IRF4 was actually bound to HSa site in non-stimulated lymphoid cells (Fig. I.R.1.5). Interferon is a well-known inducer of CD69 expression in response to infection by virus and, therefore, it is possible that IRF4 binding increases CD69 expression after cell stimulation. In addition to this TF, this analysis also revealed that HSa also functioned as a binder for HEY1, POU2F2, PAX5, BATF, PU.1, c-Myc and Pbx3 (Fig. I.R.1.3).

All these theoretical and empirical data revealed a putative regulatory role for HSa element of Intron I. These and additional evidences about regulatory elements in Intron I of CD69 have been published by our group (222) (see Appendix II).

2. ANALYSIS OF CONSERVED TRANSCRIPTION FACTOR BINDING SITES IN 5' CONSERVED NON-CODING SEQUENCES OF CD69

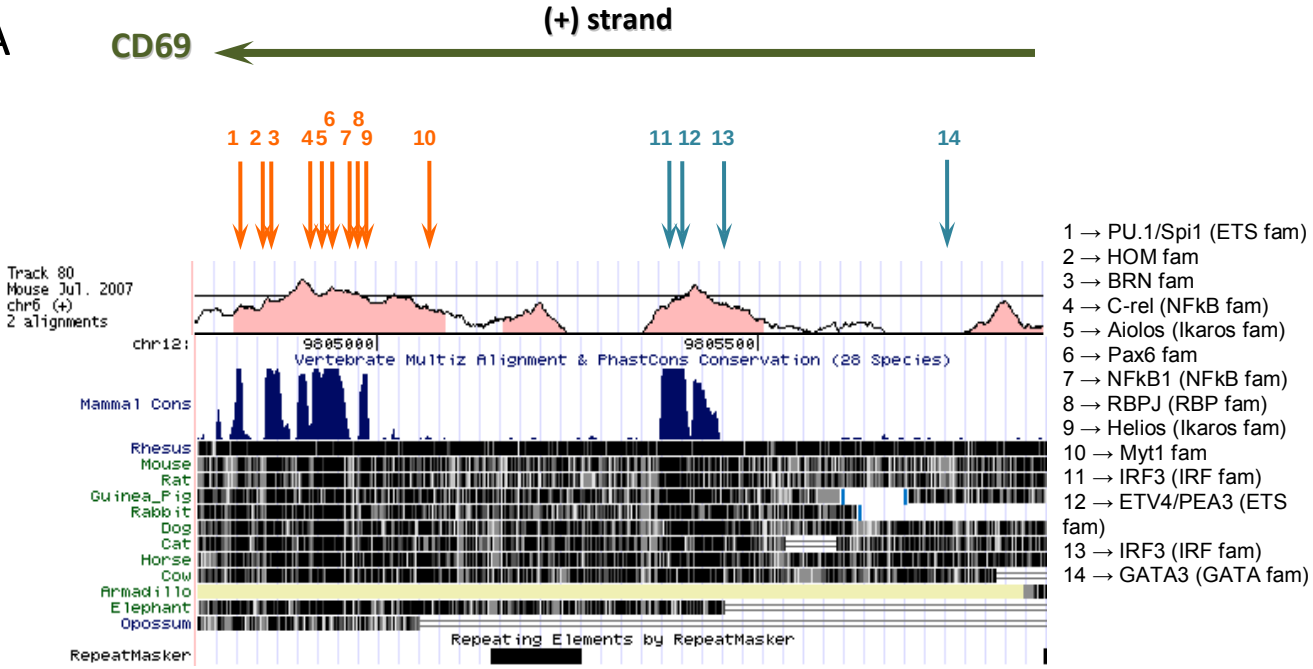
Upstream CNSs were previously reported to be important regulators of CD69 gene expression (46). To further investigate their regulatory properties we employed software tools to search for conserved transcription factor binding sites (TFBS). Using the same tools as for Intron I, we compared sequences of human, mouse, rat, rhesus, dog and horse, obtaining the plots shown in Appendix I.

For better comprehension, raw data from Appendix I was displayed in Figure I.R.2.1A (CNS1 plus promoter), B (CNS2), C (CNS3) & D (CNS4). Positions of the conserved TFBSs are depicted as colored arrows over a track of human-mouse conservation from VISTA Browser (*top*). At the bottom, a plot of mammal conservation from USCS Browser is depicted.

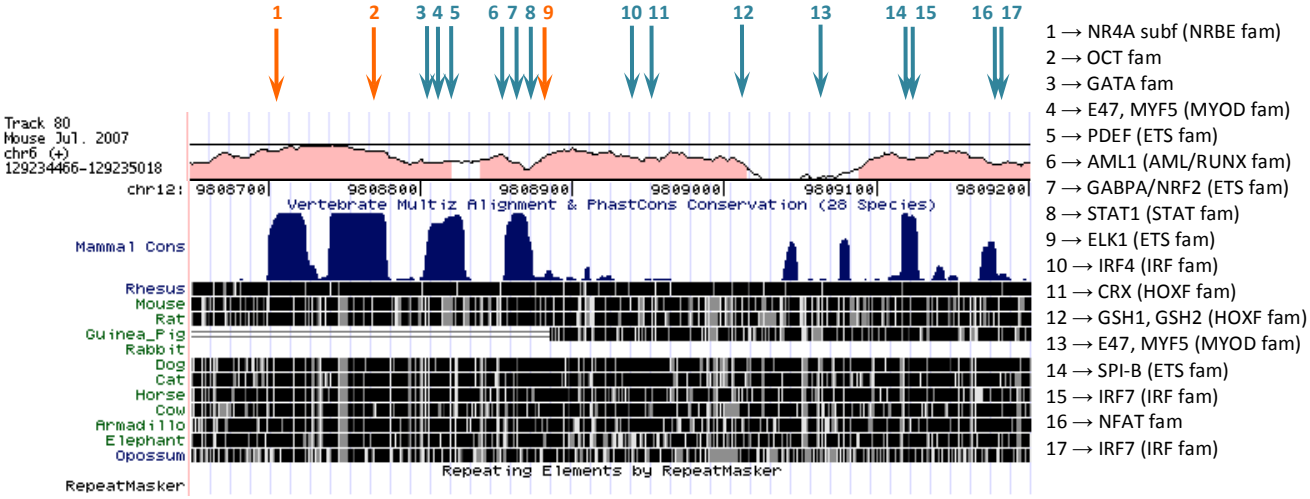
Numerous conserved TFBSs with immune-related function have been found in the four CNSs (Fig. I.R.2.1, top). Also, when comparing the position of conserved TFBSs with mammals conservation track obtained with USCs browser (Fig. I.R.2.1, bottom), most of the TFBSs are conserved in the whole group of mammal species.

In CNS1, which includes the promoter sequence (Fig. I.R.2.1A), the highly conserved TFBS are the sites located in the promoter. These include several binding sites from Ikaros, NFκB and ETS families. Concerning CNS2 (Fig. I.R.2.1B), we found more than 15 conserved TFBS, being the sites corresponding to the nuclear receptor subfamily 4 of factors (NR4A), Oct-1 and Elk-1 transcription factors the most highly conserved ones. Other relevant sites conserved in 4/5 species of the 6 analyzed were TFBSs matching to GATA, MyoD, RUNX, Nrf2/GABPA, IRF or NFAT. The most conserved TFBSs in CNS3 are found in its central part (Fig. I.R.2.1c); among them there are the lymphoid enhancer binding factor (LEF-1), the glucocorticoid receptor (GR), RUNX and Forkhead Box (FOX) families. Despite being a shorter sequence, CNS4 has highly conserved TFBS, where STAT binding sites are remarkably present with 3 binding sequences, and also locations for NFAT and Bcl-6 (Fig. I.R.1.1D).

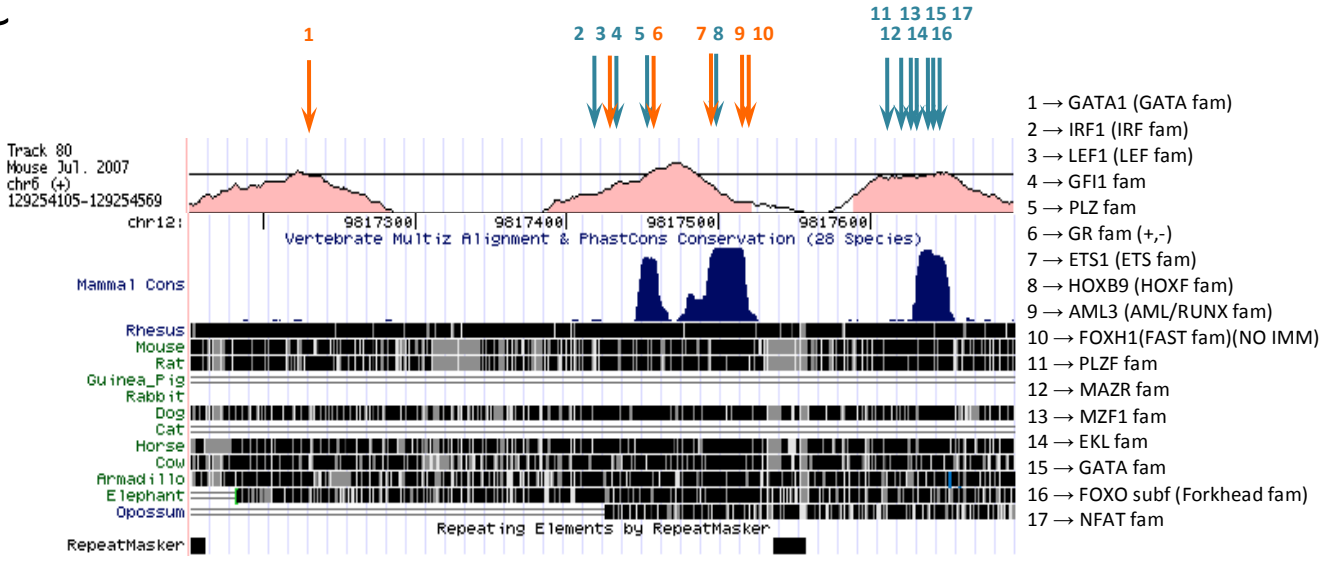
A


CD69
(+) strand
1 2 3 6 8 4 5 7 9 10 11 12 13 14
Track 80
Mouse Jul. 2007
chr6 (+)
2 alignments
chr12:
9805000
9805500
Vertebrate Multiz Alignment & PhastCons Conservation (28 Species)
Mammal Cons
Rhesus
Mouse
Rat
Guinea_Pig
Rabbit
Dog
Cat
Horse
Cow
Armadillo
Elephant
Opossum
Repeating Elements by RepeatMasker
RepeatMasker
1 → PU.1/Spi1 (ETS fam)
2 → HOM fam
3 → BRN fam
4 → C-rel (NFkB fam)
5 → Aiolos (Ikaros fam)
6 → Pax6 fam
7 → NFkB1 (NFkB fam)
8 → RBPJ (RBP fam)
9 → Helios (Ikaros fam)
10 → Myt1 fam
11 → IRF3 (IRF fam)
12 → ETV4/PEA3 (ETS fam)
13 → IRF3 (IRF fam)
14 → GATA3 (GATA fam)

B


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Track 80
Mouse Jul. 2007
chr6 (+)
129234466-129235018
chr12:
9808700
9808800
9808900
9809000
9809100
9809200
Vertebrate Multiz Alignment & PhastCons Conservation (28 Species)
Mammal Cons
Rhesus
Mouse
Rat
Guinea_Pig
Rabbit
Dog
Cat
Horse
Cow
Armadillo
Elephant
Opossum
Repeating Elements by RepeatMasker
RepeatMasker
1 → NR4A subf (NRBE fam)
2 → OCT fam
3 → GATA fam
4 → E47, MYF5 (MYOD fam)
5 → PDEF (ETS fam)
6 → AML1 (AML/RUNX fam)
7 → GABPA/NRF2 (ETS fam)
8 → STAT1 (STAT fam)
9 → ELK1 (ETS fam)
10 → IRF4 (IRF fam)
11 → CRX (HOXF fam)
12 → GSH1, GSH2 (HOXF fam)
13 → E47, MYF5 (MYOD fam)
14 → SPI-B (ETS fam)
15 → IRF7 (IRF fam)
16 → NFAT fam
17 → IRF7 (IRF fam)

C


1 2 3 4 5 6 7 8 9 10 11 13 15 17 12 14 16
Track 80
Mouse Jul. 2007
chr6 (+)
129254105-129254569
chr12:
9817300
9817400
9817500
9817600
Vertebrate Multiz Alignment & PhastCons Conservation (28 Species)
Mammal Cons
Rhesus
Mouse
Rat
Guinea_Pig
Rabbit
Dog
Cat
Horse
Cow
Armadillo
Elephant
Opossum
Repeating Elements by RepeatMasker
RepeatMasker
1 → GATA1 (GATA fam)
2 → IRF1 (IRF fam)
3 → LEF1 (LEF fam)
4 → GFI1 fam
5 → PLZ fam
6 → GR fam (+,-)
7 → ETS1 (ETS fam)
8 → HOXB9 (HOXF fam)
9 → AML3 (AML/RUNX fam)
10 → FOXH1(FAST fam)(NO IMM)
11 → PLZF fam
12 → MAZR fam
13 → MZF1 fam
14 → EKL fam
15 → GATA fam
16 → FOXO subf (Forkhead fam)
17 → NFAT fam

D

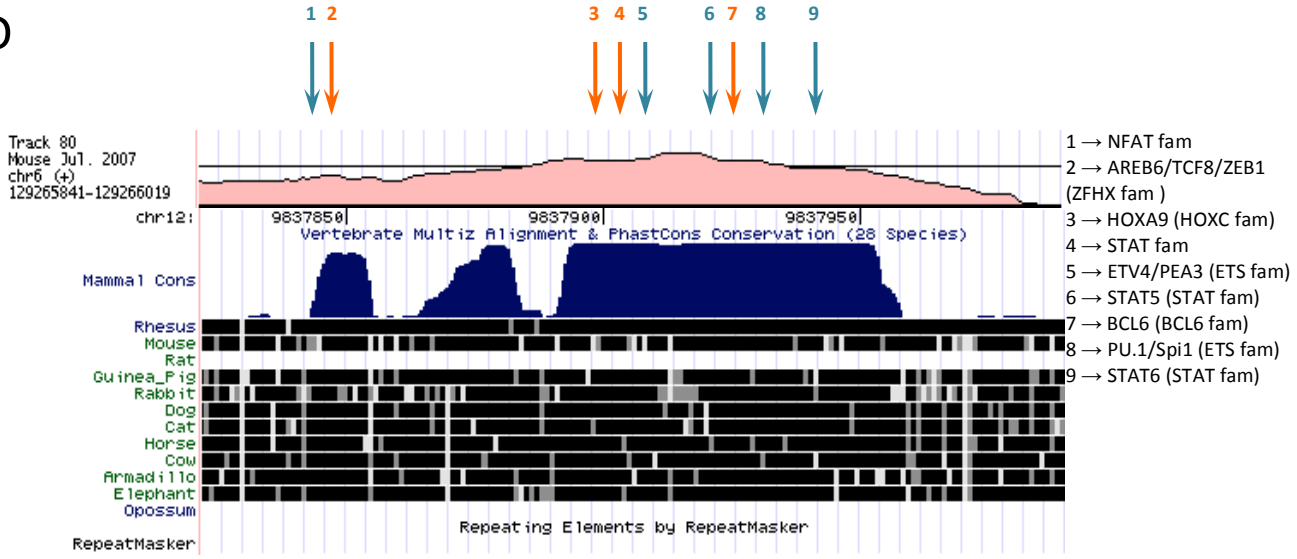


Fig. I.R.2.1. Identification of consensus transcription factor binding sites in the 4 CNSs of 5' non-coding sequence of CD69 Locus.(a) Promoter and CNS1; (b) CNS2; (c) CNS3; (d) CNS4. *Top,* VISTA plot analyses between human and mouse CNSs in the CD69 locus. Human sequences are shown on the x axis and percentage similarity to mouse on the y axis. *Bottom,* VISTA track representation of CNSs conservation among mammals (plot) and species indicated (black, most conserved zones; grey, less conserved; white, non-conserved). *Repeatmasker* indicates the position of repetitive zones in black. Above top, colored arrows mark the consensus transcription factor binding sites (long arrows: TFBS conserved for 6 species –human, mouse, rat, rhesus, dog, horse - ; short arrows: TFBS conserved for 4 or 5 species of those species). This TFBS are founded using *Genomatix DiAlign* (see *Material & Methods* and *Appendix I*). Numbered legend on right. Subfamilies of transcription factors are referred to all the sequences in case the unique TFBS was identical for all the sequences. In case the TFBS correspond to the same family but not the same subfamily, only human TF is shown. In brackets, TF family of each factor.

We compared predicted data with empirical data of ChIP-seq from ENCODE consortium for the four CNSs using the same way that we formerly analyzed Intron I. For the cells lines and transcription factors studied, CNS2 is a region remarkably rich in TFBS, even more than the promoter (Fig. I.R.2.2). In contrast, CNS1, CNS3 and CNS4 seem to be much less relevant in TF binding. Only CNS4 presents TFBS for *c-jun*, *c-fos*, GATA-2, *junD*, p300 and CEBPB in non-hematopoietic lines and for SRF and the Myocyte-specific enhancer factor 2A (MEF2A) in hematopoietic lines. These TFBS are located in the region of highest homology in CNS4 (Fig. I.R.2.3). Upstream CNS4 there is a region with high abundance of TFBS, most likely to be an insulator due to the various evidences of CTCF binding, a protein involved in transcription insulation (223-225).

The vast majority of TFBS in the promoter and the CNS2 have been found in only the hematopoietic lines: G12878 (B-lymphocyte/lymphoblastoid) and K562 (myeloid precursor); whereas CNS4 shows more

ubiquitous binding sites (Figs. I.R.2.2 & I.R.2.3). This suggests an specific role for CNS2 in hematopoietic regulation of CD69 transcription, as previously shown in T cell activation *in vitro* (46).

Among the most tightly bound transcription factors to CNS2, there are GATA-1, *c-jun*, NFκB, SRF and TCF12; among them, GATA, SRF and TCF12 (which belongs to MyoD family) binding sites were

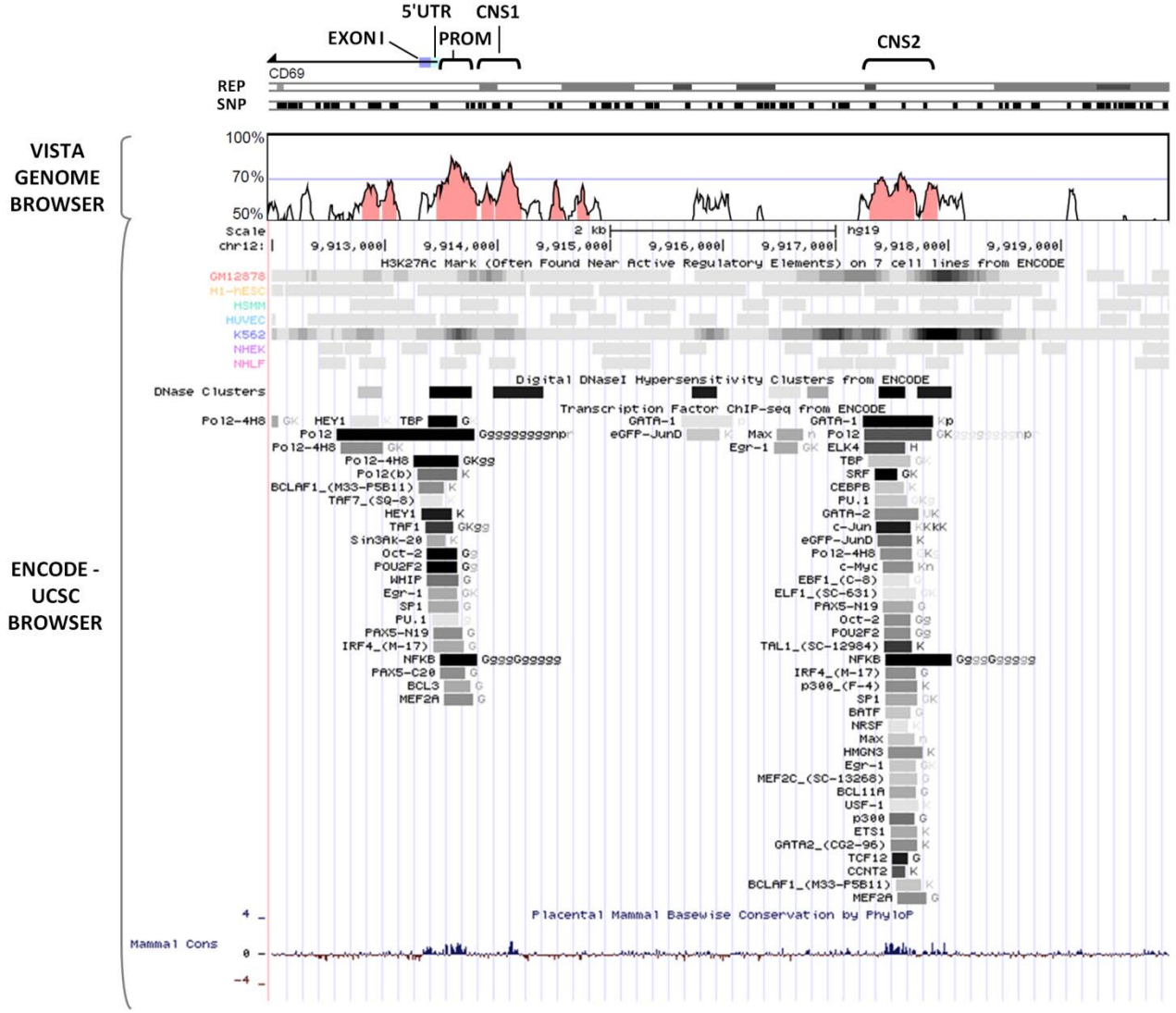


Fig. I.R.2.2. ENCODE data for promoter, CNS1 and CNS2 of human CD69. Data from ENCODE is depicted through UCSC browser for (from top to bottom): histone marks for regulatory elements (H3K27), DNase I HS, TFBSs obtained from ChIP, conservation curve for mammals (not from ENCODE). Names of the cells lines are shown for histone plot and TF ChIP-seq. The latter marks every TFBS with the name of the TF and a letter which symbolizes the cell line: G/g, GM12878 and related lines; K, K562; n, NHEK and related lines, p, PANC-1 and related. Darker bars indicate the strongest signals for every analysis. *Top*, VISTA plot of conservation human (base) to mouse sequences, where curve shows percentage of conservation (left); red zones, conserved non-coding sequences (CNSs), light blue bar on gene transcription line (top left), 5' UTR; purple bar, Exon I of CD69, and top bars, repeated sequences (REP) and single-nucleotide polymorphisms (SNPs). Base genome sequence: Human Feb. 2009, chr12 9.912.000-9.920.000.

predicted by software (Fig. I.R.2.1B). In addition, binding sites for Ets family, IRF4 and Oct, with medium binding signal in ChIP Seq (Fig. I.R.1.2), are also predicted to bind CNS2 (Fig. I.R.2.1B).

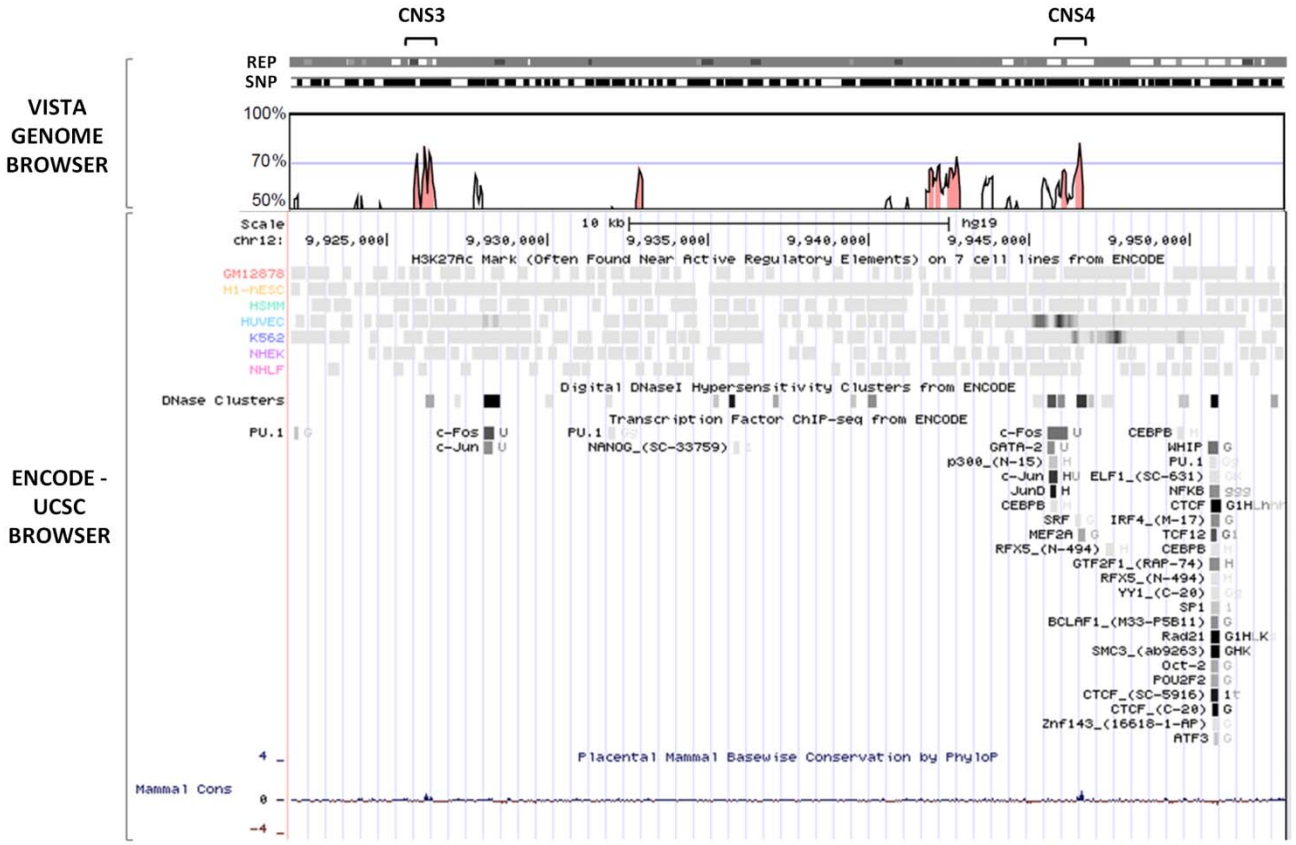


Fig. I.R.2.3. ENCODE data for CNS3 and CNS4 of human CD69. *Top*, VISTA plot of conservation human (base) to mouse sequences, where curve shows percentage of conservation (left); red zones, conserved non-coding sequences (CNSs), and top bars, repeated sequences (REP) and single-nucleotide polymorphisms (SNPs). Base genome sequence: Human Feb. 2009, chr12 9.922.000-9.953.000. *Bottom*, data from ENCODE is depicted through UCSC browser for (from top to bottom): histone marks for regulatory elements (H3K27), DNase I HS, TFBSs obtained from ChIP, conservation curve for mammals (not from ENCODE). Names of the cells lines are shown for histone plot and TF ChIP-seq. In the TF ChIP-seq data, TFBS appears with the name of the TF and a letter which symbolizes the cell line: *1*, H1-hESC; *G/g*, GM12878 and related lines; *H*, HeLa-S3 stimulated with IFNγ; *h*, HEK293 and related; *K*, K562; *n*, NHEK and related; *p*, PANC-1 and related; *t*, T-47D; *U*, HUVEC. Darker bars indicate stronger signal for each analysis.

Remarkably, NFκB, SRF and MEF2A binding are very frequent in the promoter, the CNS2 and the CNS4, but this is not surprising, as they are known to be involved in response to different stimuli that aldo induce CD69 (Figs. I.R.2.2 & I.R.2.3).

To summarize, data obtained from whole genome analyses shows potent evidence that, apart from promoter, CNS2 possess the features that may confer it to be a relevant element in the transcription regulation of CD69.

3. FUNCTIONAL STUDIES OF TRANCRIPTION FACTOR BINDING TO CNS2 IN REGULATION OF CD69 TRANSCRIPTION

The putative importance of the role of CNS2 in transcription regulation of CD69 was previously described in (46) and in Section I.R.2. A more profound analysis was performed thereafter in order to establish the relevant transcription factors that control CD69 expression.

As a first approach, conserved TFBS were studied in detail by deleting specific sequences from 51 to 166 nt in CNS2-Prom-luciferase reporter vectors (*pPr2*). Four variants of *pPr2* were generated: *pPr2ΔA*, *pPr2ΔB*, *pPr2ΔC* and *pPr2ΔD* (Fig. I.R.3.2). Each one lacks some conserved TFBS (see Fig. I.R.3.1 & Table I.R.3.I), grouped in clusters A, B, C & D. Afterwards, double deletions and mutated plasmids were produced (Fig. I.R.3.1 & Tables I.R.3.I, I.R.3.II).

Jurkat, K562 and U937 cell lines were used as targets of transfection, as they were established from human leukemic cells. As a first approach, we decided to transfect the luciferase vector in Jurkat cells, a CD4(+) T-cell leukemia cell line (226, 227) which has an inducible expression of CD69 (Fig. I.R.3.3A): low basal expression and high after stimulation with phorbol esters, like T-cells from human blood (67). Luciferase activities of *pPr2ΔA, pPr2ΔB, pPr2ΔC, pPr2ΔD luc* vectors transfected in Jurkat are shown in Figure I.R.3.4**.** The major effect in transcription is produced in the *pPr2ΔB* transfection, not only in basal state but also in PMA/Io or αCD3/αCD28 stimulated cells, reaching a mean luciferase activity reduction of 43% in more than 5 experiments. Decrease of *pPr2ΔA luc* activity was noted but was slighter, as an average reduction of 17% in 3 experiments. *Luc* activity of *pPr2ΔC.3* and *pPr2ΔD* was rough the same as complete plasmid *pPr2*.

Time course stimulations of other cell lines were assayed (Fig I.R.3.3B) to check their CD69 expression. K562 is a myeloid leukemia cell line with evidence to be derived from very early myeloid precursors. K562 cells are undifferentiated blast cells which lack monocytic and granulocytic cytoplasmic characteristics and lymphoid markers, but have receptors for the constant fraction of immunoglobulins (Fc receptors) (228) and can be differentiated to megakaryocytes in presence of PMA (229, 230). The U937 cell line was established from an histiocytic lymphoma, so its cells have the potential to differentiate into monocytes and macrophages (231, 232). Despite their differences with the Jurkat cell line, similar results were obtained with the previous plasmids transfected into U937 and K562 cell lines (Fig. I.R.3.6A, B, respectively).

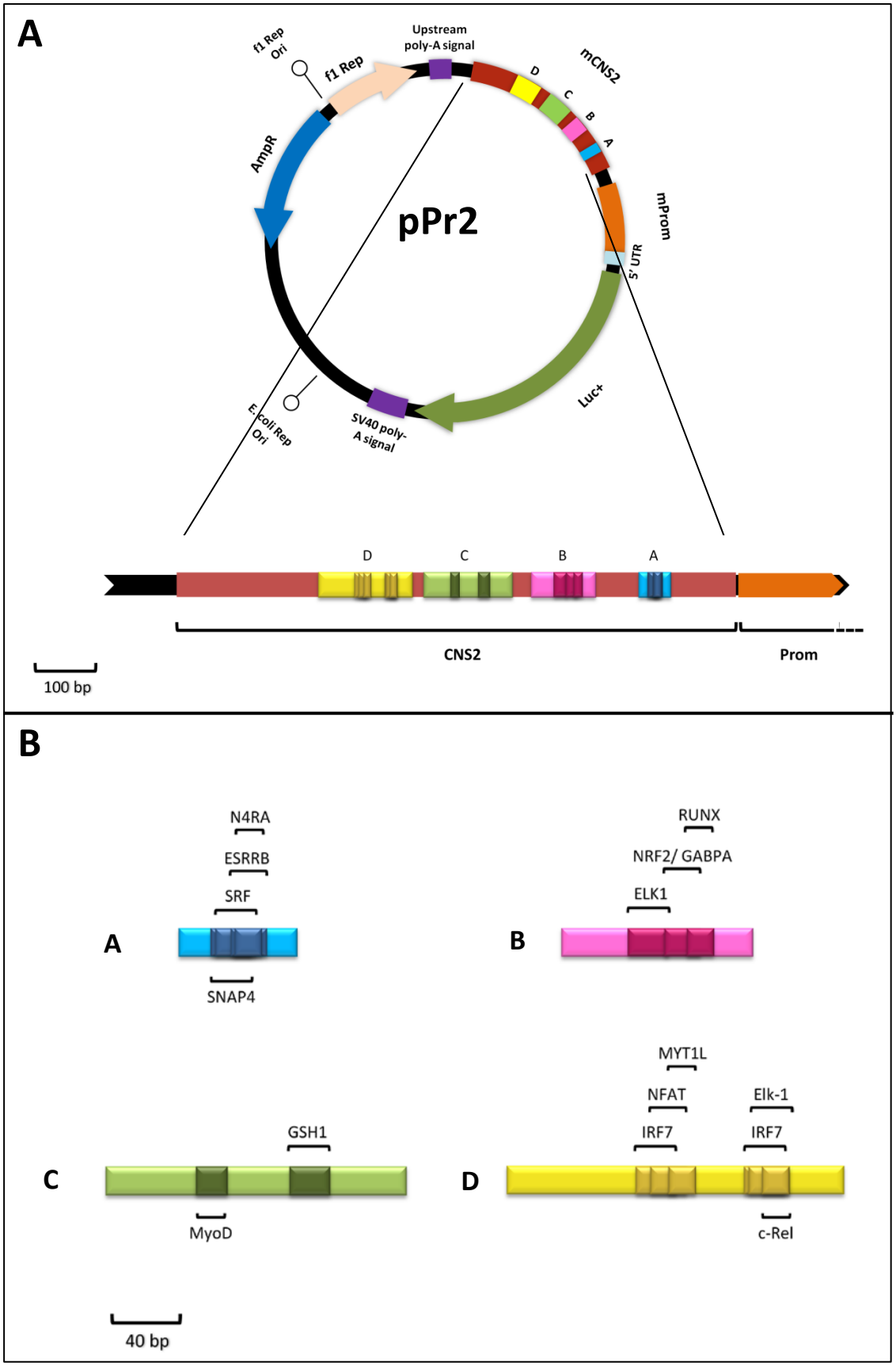


Fig. I.R.3.1. Conserved TFBSs in CNS2. A) pPr2 structure (top), clusters of common TFBSs defined in CNS2 (bottom). B) Detailed location of conserved TFBS considered in the four clusters defined in CNS2 (named as A, B, C & D, and colored as defined in *A)*).

Table I.R.3.I. Conserved Transcription Factor Binding Sites (TFBS) presence in generated luc vectors.

CLUSTER	I					II			III		IV				
TFBS	SNAP4	SRF	ESRRB	NR4A	OCT1	ELK1	GABPA	RUNX	MyoD	GSH1	IRF7	NFAT	MYT1L	ELK1	IRF7
pPr	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
pPr2	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
pPr2ΔA	×	×	×	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
pPr2ΔB	✓	✓	✓	✓	✓	×	×	×	✓	✓	✓	✓	✓	✓	✓
pPr2ΔC	✓	✓	✓	✓	✓	✓	✓	✓	×	×	✓	✓	✓	✓	✓
pPr2ΔD	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	×	×	×	×
pPr2ΔAΔB	×	×	×	×	×	×	×	×	✓	✓	✓	✓	✓	✓	✓
pPr2ΔBΔC	✓	✓	✓	✓	✓	×	×	×	×	×	✓	✓	✓	✓	✓
pPr2ΔBΔD	✓	✓	✓	✓	✓	×	×	×	✓	✓	×	×	×	×	×
pPr2.mutR	✓	✓	✓	✓	✓	✓	✓	MUT	✓	✓	✓	✓	✓	✓	✓
pPr2. mutG	✓	✓	✓	✓	✓	✓	MUT	✓	✓	✓	✓	✓	✓	✓	✓
pPr2. mutGmutE	✓	✓	✓	✓	✓	MUT	MUT	✓	✓	✓	✓	✓	✓	✓	✓
pPr2. mutS	✓	MUT	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
pPr2. mutSmutR	✓	MUT	✓	✓	✓	✓	✓	MUT	✓	✓	✓	✓	✓	✓	✓
pPr2mut.SmutRmutG	✓	MUT	✓	✓	✓	✓	MUT	MUT	✓	✓	✓	✓	✓	✓	✓

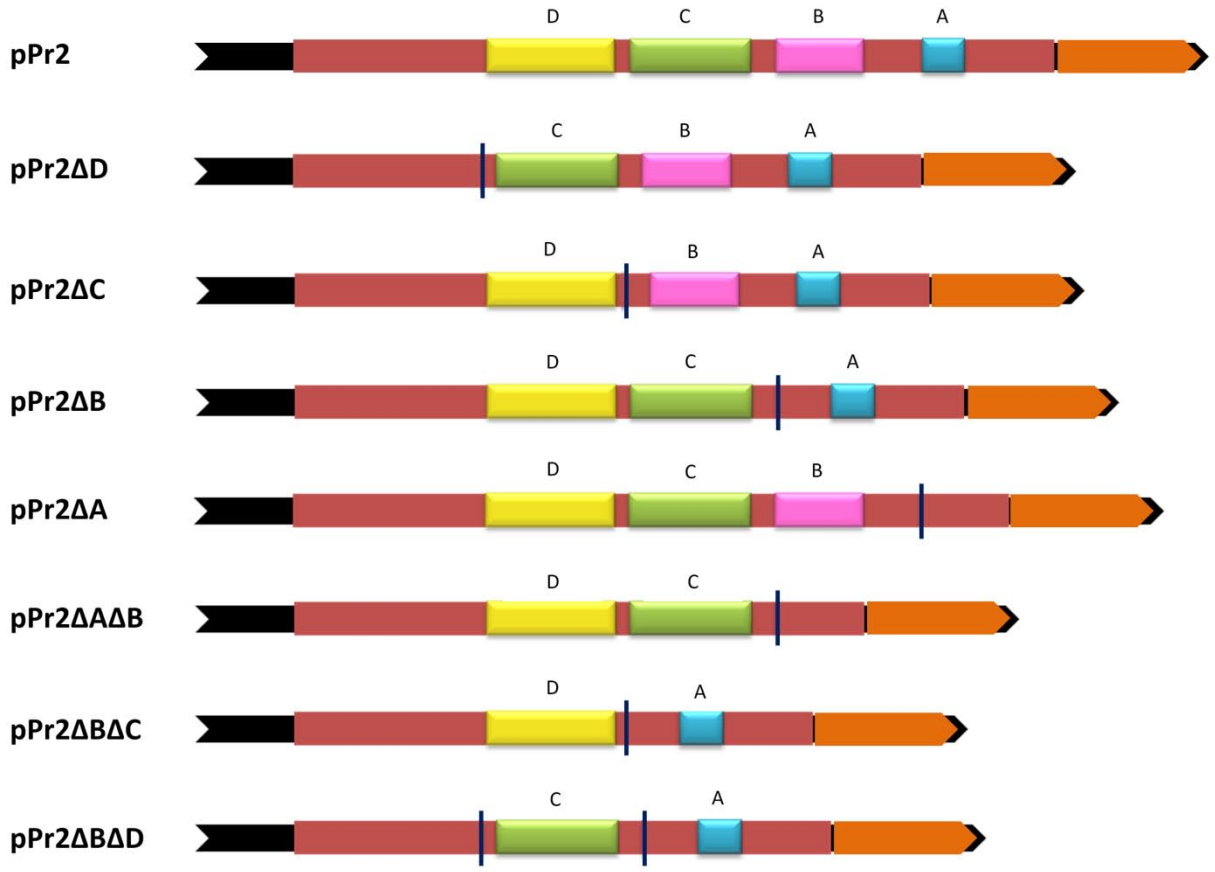


Fig. I.R.3.2. Cluster deletion in CNS2 for pPr2 plasmid. Original mCNS2- mCD69 Promoter- luc vector (top) was modified by overlap PCR and subcloning. Clusters of conserved TFBSs previously defined (Fig. I.R.3.1) were deleted individually (*pPr2ΔA*, *pPr2ΔB*, *pPr2ΔC* and *pPr2ΔD*) or in combination (*pPr2ΔAΔB*, *pPr2ΔBΔC*, *pPr2ΔBΔD*). *Red*, mCNS2 sequence; *orange*, 5' end of mCD69 promoter.

Table I.R.3.II. Site-directed mutations produced in conserved TFBS.

Plasmid Name	Mutated TFBS	5' -> 3' Sequence (CORE)	Mutated Sequence
pPr2.mutR	RUNX	GTT**TGTGGT**TCCTTT	GTT**TGT**TT**T**TCCTTT
pPr2.mutG	GABPA	CAGGT**CGGAAG**TTTGTGGT	CAGGT**C**TT**AAG**TTTGTGGT
pPr2.mutGmutE	ELK1 + GABPA	TTGTCACC**GGAA**ACTGCCTCAGGT **CGGAAG**TTTGTGGT	TTGTCACC**GGAA**ACTGCCTCACCT**C GGAAG**TTTGTGGT
pPr2.mutS	SRF	TTCT**TCATATAAGG**TCACA	TTCT**TGGTATAAGG**TCACA
pPr2.mutSmutR	RUNX + SRF	GTT**TGTGGT**TCCTTT// TTCT**TCATATAAGG**TCACA	GTT**TGT**TT**T**TCCTTT// TTCT**TGGTATAAGG**TCACA
pPr2.mutSmutRmutG	GABPA + RUNX + SRF	CAGGT**CGGAAG**TTT**TGTGGT**TCCT TT// TTCT**TCATATAA**GGTCACA	CAGGT**C**TT**AAG**TTT**TGT**TT**T**TCCTTT // TTCT**TGGTATAA**GGTCACA

[1] Mutated plasmid in Cluster A but in no TFBS, just by SRF BS (depicted in lower case).

According to the effect of deletion of *Cluster B,* this cluster appears to be relevant for expression induction, but its absence does not abolish all the enhancement activity of CNS2. Thus, the importance of RUNX, GABPA/NRF2 and Elk-1, whose binding sites are deleted in *pPr2ΔB*, were be further studied.

As the absence of Clusters A and B diminished *luc* activity, we decided to generate double deletion of clusters, combining the absences of Cluster B with Cluster A, C or D. As expected, double deletion of Clusters A and B (*pPr2ΔAΔB*) reduced the luciferase activity, to the extent of *pPr* levels (plasmid with CD69 promoter but without CNS2 enhancer) (Fig. I.R.3.5).

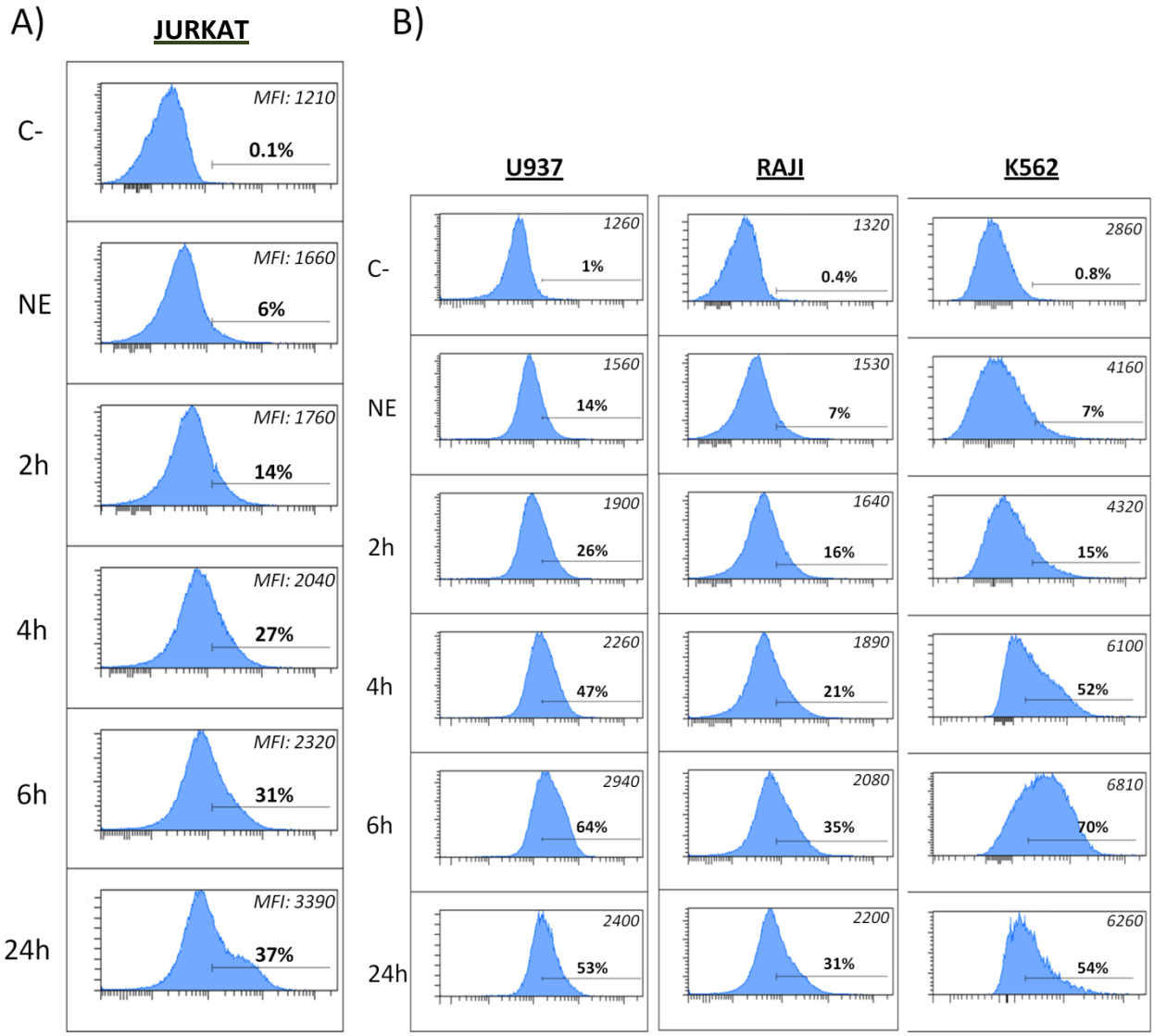


Fig. I.R.3.3. CD69 expression in human cell lines. 10^6 cells/well were stimulated with 10 ng/ml of PMA and 500 ng/ml of Ionomicin or PMA alone, or not stimulated (C-, sample non-stimulated & non-stained; NE, sample non-stimulated) in Jurkat cell line (*A*)) and U937, RAJI, K562 cells (*B*)). Percentages of CD69 positive cells (over the bar, bold) and mean fluorescence intensity (top-right corner of each plot, cursive) are depicted.

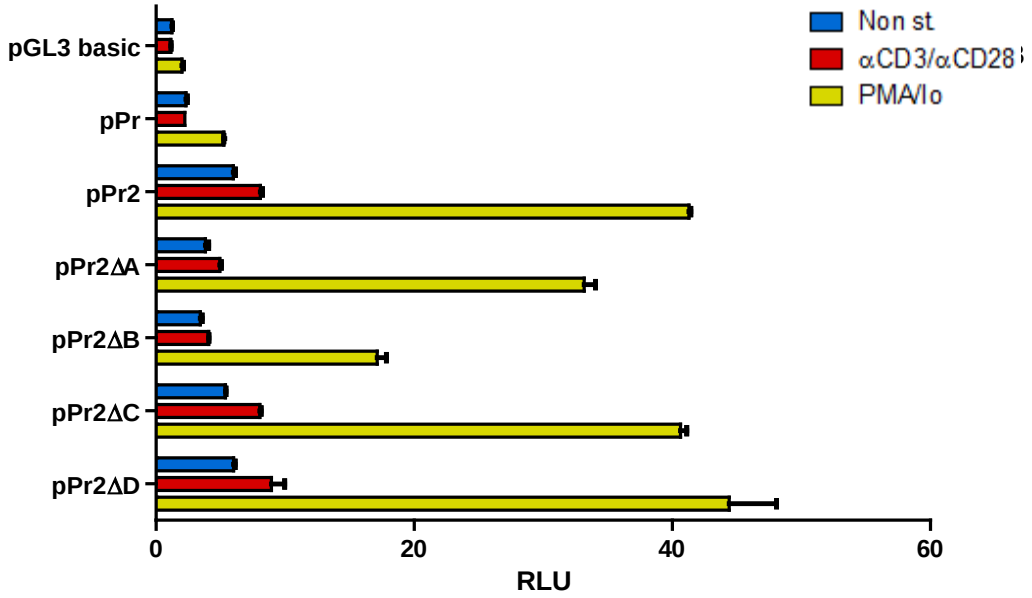


Fig. I.R.3.4. Luciferase activity of pPr2 plasmid with different deletions in CNS2. Deletions of clusters of conserved TFBSs in CNS2 of *pPr2*: *pPr2ΔA*, *pPr2ΔB*, *pPr2ΔC*, *pPr2ΔD* (see Fig. I.R.3.2) were generated and transfected into Jurkat cells and luciferase activity was measured. Basic luc-plasmid from Promega (*pGL3 basic*) and basic primer directed by mCD69 promoter (*pPr*) transfections were assayed to use as controls. Error bars are plotted as SEM of triplicates. Data are representative of at least 2 experiments. *RLU*, Relative Luciferase Units.

A different strategy was performed to determine the enhancer capacity of Clusters A and B. We cloned Cluster B and the combination of Clusters A & B upstream promoter sequence in *pPr* plasmid (generating *pPr2.A* and *pPr2.AB*, respectively) and assayed for their *luc* activity. Remarkably, Cluster B was unable to

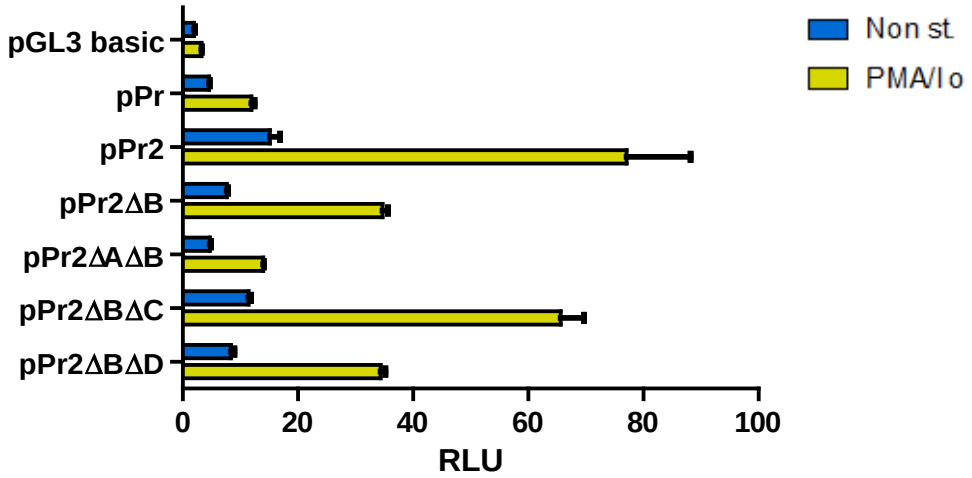


Fig. I.R.3.5. Luciferase activity of pPr2 plasmid with combined deletions in CNS2. Jurkat cells were transfected with *pPr2* plasmid with cluster B deletion combined with A (*pPr2ΔAΔB*), cluster C (*pPr2ΔBΔC*) or cluster D (*pPr2ΔBΔD*), then luciferase activity was measured. Error bars are plotted as SEM of triplicates. Data are representative of at least 2 experiments. *RLU*, Relative Luciferase Units.

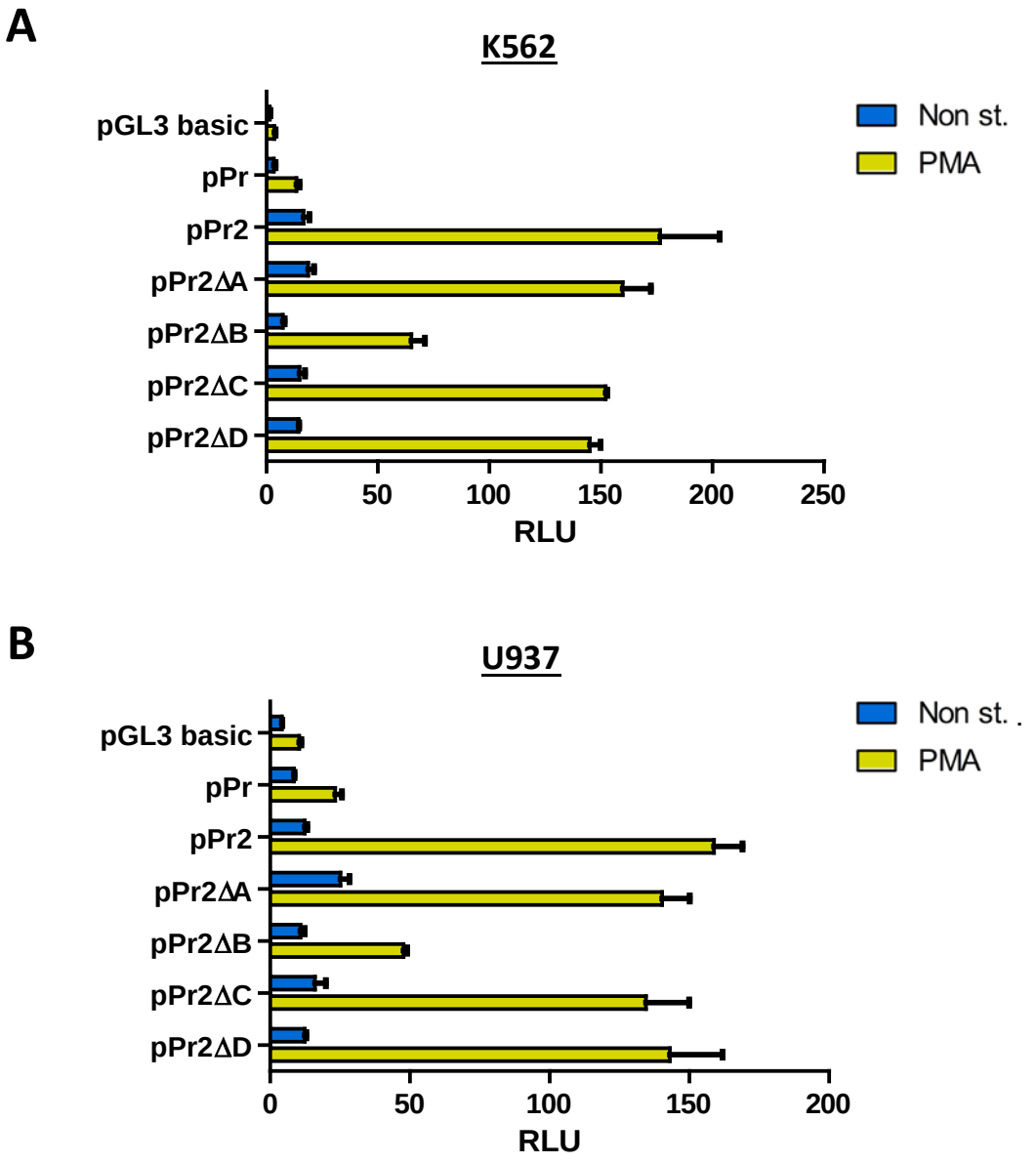


Fig. I.R.3.6. Luciferase activity of pPr2 plasmid with different deletions in CNS2. Same procedure as in Fig. I.R.3.4, except of transfecting in different cell lines: *A)* K562, *B)* U937. Error bars are plotted as SEM of duplicates. Data are representative of 3 experiments. *RLU*, Relative Luciferase Units.

induce promoter activity by its own, whereas luciferase activity of *pPr2.AB* was similar to the plasmid with complete CNS2 sequence, indicating a cooperative effect of transcription factors from Cluster A and Cluster B to product the enhancement of CD69 transcription *in vitro* (Fig. I.R.3.7).

To further investigate the role of transcription factors in CD69 regulation, *pPr2* was mutated in several TFBS of Clusters A & B of CNS2: Runx (*pPr2.mutR*), GABPA (*pPr2.mutG*, core union sequence; *pPr2.mutGmutE*, outside consensus core of GABPA and Elk1 but inside both binding sequences), SRF

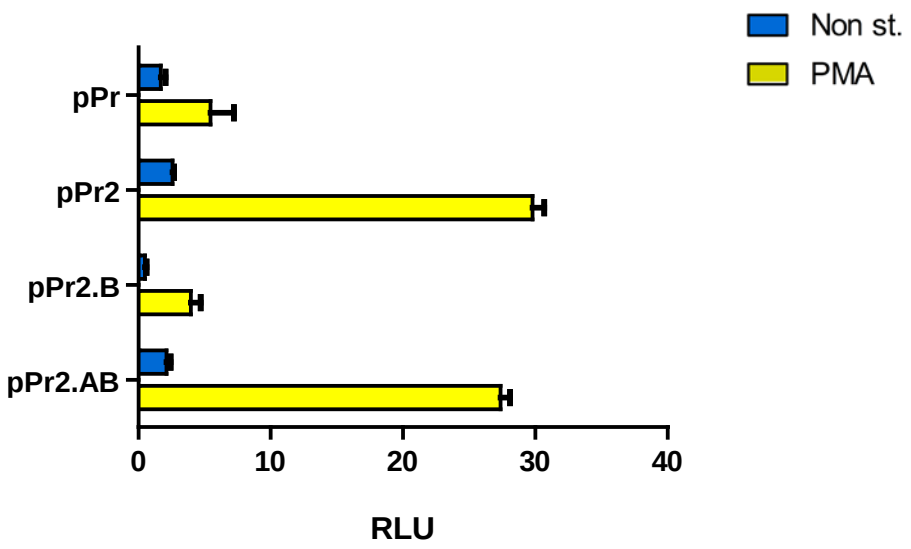


Fig. I.R.3.7. Luciferase activity of *pPr* plasmid with Clusters A & B from CNS2. Jurkat cells were transfected with *pPr2* plasmid with Cluster B (*pPr2.B*) or Cluster A plus Cluster B (*pPr2.AB*) upstream CD69 promoter and luciferase activity was measured. Error bars are plotted as SEM of triplicates. Data are representative of at least 2 experiments. *RLU*, Relative Luciferase Units.

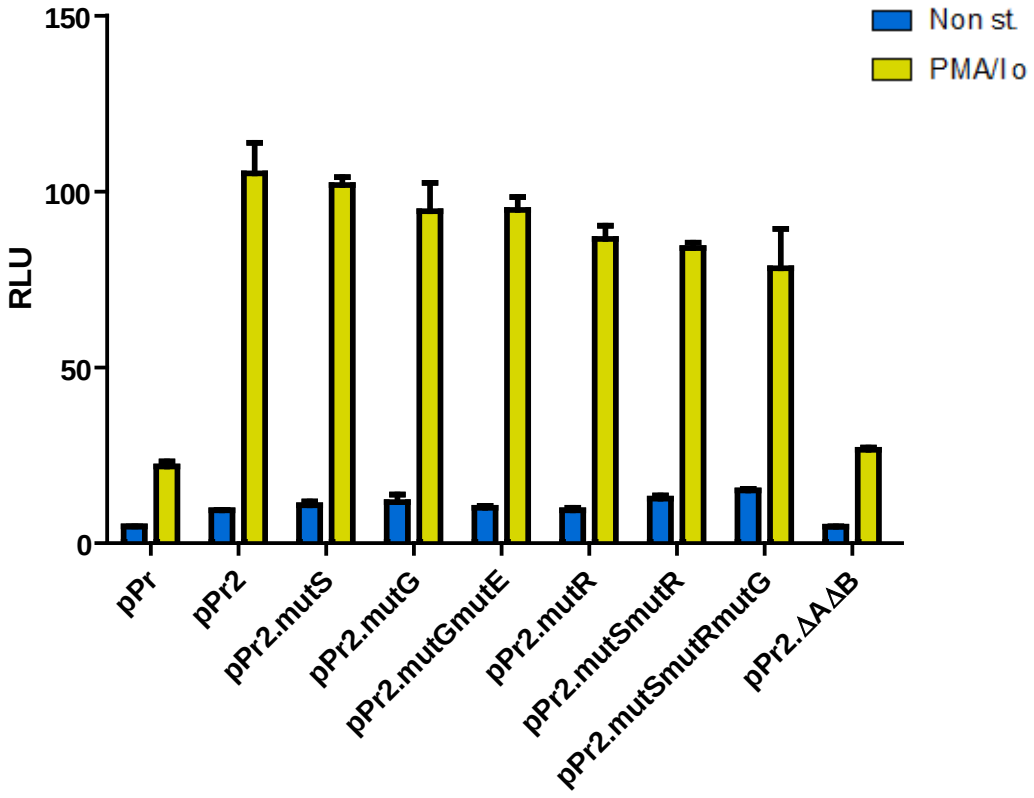


Fig. I.R.3.8. Luciferase activity of pPr2 plasmid with directed mutations in conserved TFBS of CNS2. Site-directed mutagenesis was designed for Runx (*pPr2.mutR*), GABPA (*pPr2.mutG, pPr2. mutGmutE*), SRF (*pPr2.mutS*) or combinations (Runx plus SRF, *pPr2.mutSmutR*, Runx-SRF-GABPA, *pPr2.mutSmutRmutG*). *pPr2.mutCA,* plasmid with control mutation in Cluster A After mutation, transfection in Jurkat cell line was performed. Yellow bars, PMA plus Ionomicin stimulated cells. Blue bars, non-stimulated cells. Error bars are represented as SEM of triplicates. Data are representative of 3 experiments. *RLU*, Relative Luciferase Units.

(*pPr2.mutS*) and different combinations of them (*pPr2.mutSmutR*; *pPr2.mutSmutRmutG*) (see Tables I.R.3.I & I.R.3.II). Transfection of mutated constructs in Jurkat did not have such a marked effect as in previous transfections, but the mutation in RUNX binding site produces the highest reduction (18 %) in transcriptional activity, under PMA stimulation, from all the single mutations respect to wild-type CNS2 (Fig. I.R.3.8). Besides, combination of mutations in *pPr2.mutSmutR* and *pPr2.mutSmutRmutG* produced a 20% and a 26% of reduction of *luc* activity, respectively, only observable in stimulated cells (Fig. I.R.3.8). Under non-stimulatory conditions, lower differences were observed between *pPr2* and mutated plasmids, even showing higher transcriptional activity in double-mutated plasmids (Fig. I.R.3.8).

In summary, a minimum sequence of regulation of CD69 transcription in CNS2 has been found, composed by Clusters A & B. RUNX1 transcription factor is mainly responsible of the function of this core, specifically under stimulatory conditions, but requires additional elements in Cluster A, pointing to a regulation involving several transcription factors, like fine CD69 regulation is expected to involve.

4. *IN VITRO* SILENCING OF hRUNX1 ALTERS hCD69 TRANSCRIPTION

As we have found evidences of an important role of RUNX binding site in stimulation through CNS2, we analyzed the effects of inhibiting the expression of RUNX1 and RUNX3 in Jurkat cells.

Although silencing transcription of RUNX3 barely reduced CD69 transcription (data not shown), blocking RUNX1 expression did affect mRNA levels of CD69, in agreement with previous results of *luc* assays (Fig. I.R.4.1 & Fig. I.R.4.2). We first analyzed the effects of RUNX1 silencing in RNA and protein levels of CD69 after 24 hours of nucleofection. Results indicate that transitional absence of RUNX1 reduced CD69 levels of mRNA in approximately a 75% in Jurkat cells after a 24-h nucleofection (Fig. I.R.4.1A). This reduction did not correlate with a down-regulation of CD69 protein levels (Fig. I.R.4.1B), showing a slight induction by nucleofection.

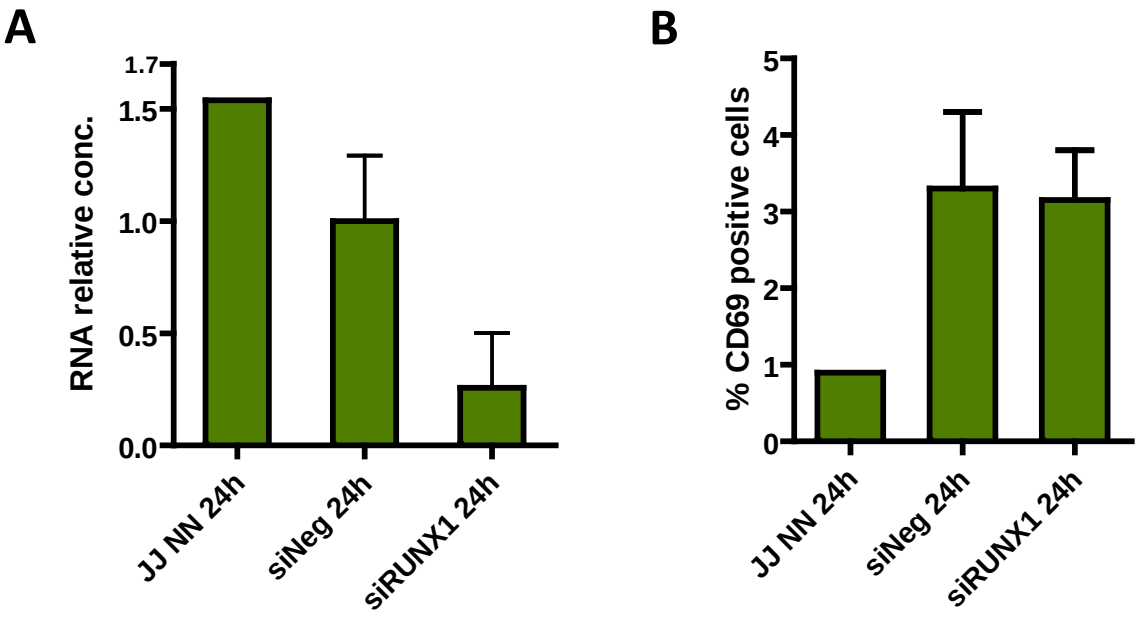


Fig. I.R.4.1. Down-regulation of hCD69 mRNA but not hCD69 protein after RUNX1 silencing. Jurkat cells were nucleofected or not (*JJ NN 24h*), with RNA silencer of RUNX1 (*siRUNX*) or a control silencer (*siNeg*) for 24 h and then, A) RNA was extracted and analyzed by Real-Time PCR or B) cells were stained with anti-hCD69 and analyzed by flow cytometry. A) RNA relative concentration was calculated using RNA from the subunit 18S of ribosomes as reference. "1" corresponds to the mean value of the 3 triplicates for siNeg transfection. Each transfection was done three times and the experiment is representative of three. Data are presented as Mean ± SEM. B) Each bar represents mean of duplicates ± SEM except for *JJ NN 24h,* which represents one sample. Results representative of two experiments.

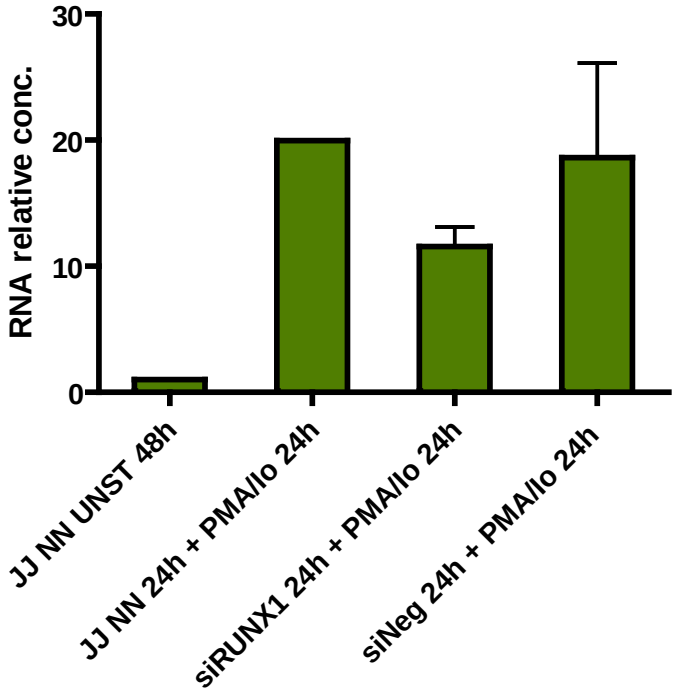


Fig. I.R.4.2. Low PMA-induced hCD69 RNA production after 24h-nucleofection of siRUNX1. Jurkat cells were nucleofected or not (*JJ NN*) with RNA silencer of RUNX1 (*siRUNX1*) or a control silencer (*siNeg*) for 24 h and then stimulated with PMA plus Ionophore (+ *PMA/Io*) or left untreated (*JJ NN UNST 48h*). After other 24 h, RNA was extracted and analyzed by Real-Time PCR. Relative concentration was calculated using RNA from the subunit 18S of ribosomes as reference. Relative concentration of "1" corresponds to the non-nucleofected & non-stimulated sample. Each transfection was done twice (except *JJ NN UNST 48h*) and this experiment is representative of a total of two. Data are presented as Mean ± SEM.

The effect in mRNA was also pronounced if cells are stimulated with PMA plus Ionomicin for 24 more hours, as the RNA levels increase considerably (Fig. I.R.4.2), even considering nucleofection was produced 48 hours before and the effect of silencing would be diminished.

Those results suggest a relatively relevant regulatory function of RUNX1 on CD69 transcription, as this is affected by RUNX1 activity in basal conditions and under stimulation. Nevertheless, correlation between RNA and protein expression levels of CD69 remains to be determined under stimulatory conditions, in order to establish whether a reduction of CD69 protein induction is occurring.

DISCUSSION

Our goals in the present study were to employ *in silico* data available online to study CD69 regulatory regions and establish experimentally the relevant elements from these regions in CD69 transcriptional regulation.

Conserved TFBS prediction permits finding *cis*-acting elements on their basis of conservation during evolution, indicating possible beneficial effects on species survival as a reason to remain almost unchanged. We found several conserved sequences that match known TFBS in the four 5' CNS and Intron I of CD69 employing the software tool *Genomatix DiAlign TF*. In this method, for each family or subfamily of transcription factors a weight matrix pattern is defined, representing the complete nucleotide statistical distribution for each single position of the binding sequence to the transcription factor (233). Therefore, we considered these data as a good start point to further analyze regulation of CD69.

By experimental procedures, we observed that Intron I of CD69 is found in open chromatin conformation, containing a potent HS site, *HSa*. These results were corroborated by data from ENCODE consortium, which is obtained by genome-wide experimentation, allowed us to obtain relevant information about DNase clusters and TFBS and their cell type specificity. HSs found in ENCODE for intron I are slightly restricted to lymphocytes (Fig. I.D.1), which correlates with DNase hypersensitivity assay results we obtained from human PBLs. The fact that HSa is open at basal state but also is inducible by PKC activators suggests that its accessibility is increased by PKC- derived signals. Remarkably, this is the first inducible HS found for CD69, as chromatin accessibility of the previously defined four 5' CNSs were found to be constitutively open in thymocytes of a transgenic line that do not express CD69 (46). This difference may be produced by thymocytes and peripheral lymphocytes presenting different regulation of chromatin accessibility; or by specific-site regulation of nucleosome disposition. We also show the presence of weak HS sites in Intron I. They are observed in both our hypersensitivity assays and in ENCODE data, and seem to disappear under lymphocyte activation. This effect could be masked by the increasing of accessibility of HSa upon activation, which would diminish the length and thus the detectability of neighboring fragment in HS assays.

We had found in previous studies that CNSs were in open chromatin conformation in mouse thymocytes (46). Data of DNA accessibility from ENCODE for different cell lines (Fig. I.D.1) confirmed that point, showing open chromatin for CNS2, CNS1 and promoter for all hematopoietic cells available, including progenitors, mature B cells, Th1, Th2, regulatory T cells (Tregs) and cell lines GM12878, Jurkat and K562. In addition, endothelial HUVEC cells show light accessibility, as low levels of transcription were detected for endothelial CD105+ cells (BioGPS database: http://biogps.org/#goto=genereport&id=969 (234-236)). These results indicate expression of CD69 in endothelial cells need to be further studied. These data points to a restricted expression of CD69 in hematopoietic lineages due to, at least in part, regulation of chromatin accessibility.

As with DNase I HSs, TF binding by ChIP for CNS2, promoter and Intron I, are found almost strictly in hematopoietic cells, including primary cells and cell lines. In contrast, data from ChIP-seq for CNS4 indicated more ubiquitously regulatory functions, suggesting that the former 3 regulatory elements are

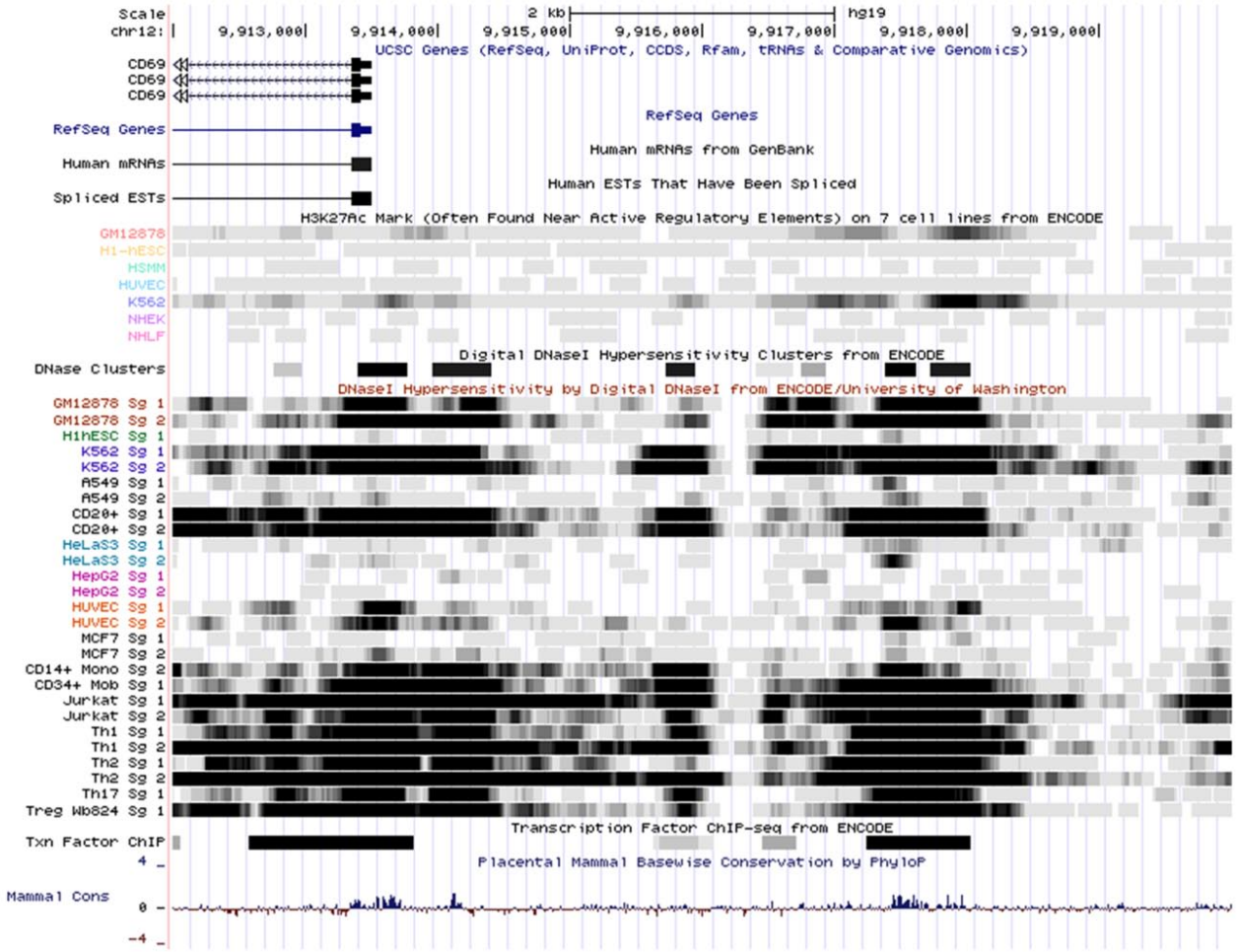


Fig. I.D.1. DNase hypersensitivity sites in intron I, promoter, CNS1 & CNS2 of CD69 for different cell lineages. ENCODE DNase I hypersensitivity track from Washington University in different cell lines indicated in left legend

responsible for hematopoietic-specific transcription regulation.

ChIP-seq from ENCODE pointed to CNS2 as an important element in CD69 regulation, as it was shown as an accessible region marked with an active histone mark for enhancers (H3K27 acetylation (237)) and with evidence of binding more than 30 transcription factors. These data confirmed our previous results *in vitro* where CNS2 was observed to be a potent enhancer of CD69 transcription and to show activation H3-acetylation histone marks in CNS2 (46). In this chapter, we show the finding of the minimum core sequence of CNS2 responsible for induction of transcription activity. This core includes the clusters A & B; A contains the conserved TFBS for RUNX, GABPA, ELK1; and B, for SRF, STAT1, SNAP4 and ESRRB. Despite that Cluster B seems to be the most important cluster for CNS2 function, as its deletion abrogates transcriptional activity in a ~50%, it is not able to induce any transcriptional activity by its own. In contrast, its association with Cluster A restores all the transcriptional induction, indicating cooperation between factors is occurring. According to this idea, evidences of interactions between SRF and Elk-1 in the *c-fos* promoter have been described (205).

RUNX1 site has been shown to be an important mediator in CD69 transcriptional induction, as its mutation in Cluster B reduced considerably luciferase activity mediated by the promoter and CNS2 of CD69, and CD69 mRNA is decreased by silencing of RUNX1 RNA. Site-directed mutagenesis reduced transcriptional activity but not to the same extent as Cluster B deletion. We consider that RUNX1 may conserve the ability to bind CNS2 after a 2-nt mutation and/or that it may have overlapping functions with other transcription factors. Our data implicating RUNX1 in CD69 upregulation is supported by evidences of reduced CD69 expression in CD4-conditional knock-out of RUNX1 (238)

According to functional *in vitro* results, we propose CD69 induction by CNS2 could be mediated by a *billboard* mechanism (26), which consists in none of the transcription factors being essential and their action being cooperative. This would explain why the whole sequence formed by Clusters A & B is required. We propose that RUNX, SRF and GABPA/Elk-1 could be forming a multiprotein complex that function as a unity but that may work also in the absence of one or more factors in a cooperative manner. We envision RUNX and GABPA TF overlap partially in their binding to this site (maybe changing positions), and could bind to SRF by DNA folding, as it was reported for other transcription factors (reviewed in (28)). As no conserved NFκB binding site was found by software prediction but NFκB binding to CNS2 has actually been found by ChIPseq, a folding mechanism of DNA could be working also between promoter

and CNS2 which allows NFκB binding to other TF that bind CNS2 directly, as was previously reported (239). To study those hypotheses, additional ChIP experiments are required, as well as chromosome conformation capture (3C) assays and pull-down assays.

In brief, CNSs and Intron I have been defined as important regulators of CD69 based in theoretical and empirical data from chromatin accessibility and transcription factor binding. Additionally, the core of Cluster A & B of CNS2 has been proved to be a potent inducer element of CD69 transcription, apart from promoter (46, 141, 145). RUNX1 is proposed to play a major role in the core function but requires the action of other transcription factors as SRF, considering it was one of the most relevant factors bound in hematopoietic cells in ENCODE data and its binding site is located in Cluster A

In contrast to the *in vitro* enhancer function, previous results in TG mice suggested an inhibitory function for CNS2, as transgene expression was inhibited when directed by CNS2-CNS1-Promoter elements (46). However, those observed *in vivo* effects could be produced by other types of regulation, like absence in the transgene of most of the non-coding sequences, including 3' UTR, introns, intergenic sequences, etc. Further studies employing whole CD69 locus may elucidate CNS2 functions in its genomic context.

CHAPTER II:

REGULATION OF CD69 EXPRESSION IN CD69-BAC-IN TRANSGENIC MODELS

CAPÍTULO II:

REGULACIÓN DE LA EXPRESIÓN DE CD69 EN MODELOS TRANSGÉNICOS CD69-BAC-IN

INTRODUCTION

Molecules involved in leukocyte activation are known to be tightly regulated, as they suffer rapid changes in their biologically active protein levels. Functionally active protein levels are regulated by plenty of mechanisms, including chromatin accessibility, modulation of transcription by transcription factors, stabilization/degradation of mRNA, or regulation of protein localization by different proteins (see Part 1 of Introduction). A well known model of complex gene regulation in that type of genes is the cytokine produced after T-cell activation IL-2, which is controlled by both transcriptional and posttranscriptional events. It has been proved that in IL-2 locus there are *cis*-acting elements which bind the transcription factors NFκB, Oct, NFAT, that markedly control the promoter function (reviewed in (240)). Additionally, several studies proposed an mRNA stabilization mechanism induced by activation stimuli (241-243). Another example is Bcl-6, a transcription factor involved in B cell differentiation, which is down-regulated after B cell activation, and is also controlled by posttranscriptional mechanisms (244, 245).

CD69, an inducible receptor immediately expressed after lymphocyte activation which suffers rapid changes in its mRNA and protein levels (246), is expected to present different mechanisms of expression regulation. Regarding its transcriptional regulation, several *cis*-acting elements have been described. Among them, the promoter (142) and CNS2 (46) are potent inducers of transcription, and present different binding elements of relevant transcription factors *in vitro*, as NFκB (141), AP-1 (145) and RUNX (Chapter I). However, the CNS2 function when is located in its genomic context remains to be determined, as TG mouse lines whose transgene was directed by the CNS2-CNS1-promoter construct showed a reduced expression of the transgene compared to the lines with the transgene under solely the promoter (46).

As conventional reporter mice transgenic for *cis*-regulatory elements lines carry only a small fraction of these elements and they are isolated from their genomic environment, a new generation of TG lines has been developed during the last 15 years. The new transgenes for this purpose are made with the insertion into the chromosomal DNA of large pieces of genome (100-200 Kb approx.) with the use of Bacterial Artificial Chromosomes (BACs) (247). BACs are DNA constructs based on functional fertility plasmids (F-plasmids) with some modifications to facilitate the cloning and the transformation of large fragments of genomic DNA (248, 249). F-plasmids may contain up to 1 Mb of DNA, are highly stable and one or two

copies can be found in a bacterial cell, being little prone to produce recombination with genomic bacterial DNA (250).

Modification of BACs by homologous recombination and subsequent transgenesis has been proved to be a powerful means to generate tools for the study of gene expression and function *in vivo* and *in vitro* (251-258), due to the resemblance to the endogenous locus. Conventional transgenes usually are composed by cDNAs, tag genes, or even unproductive target genes, lacking regulatory elements as introns or UTR ends and distal regulatory elements. They also are driven by unrelated ubiquitous or tissue-specific promoters. All those features do not reproduce the endogenous ambient of regulation.

BACs transgenesis can not only be used to generate TG mice (259, 260), but also to produce stably transfected cell lines (261, 262). These procedures allow various analysis, i.e. expression of a molecule in specific tissues (259, 263-265), protein location inside cells (262) or effects of *cis*-acting elements in the regulation of gene expression (266, 267), through the introduction of reporters in the BACs.

BAC-in transgenic mice have been used in several studies of regulation of immune-related genes, as RAG2 (255, 268), Pax5 (269) or *Ifng* locus (45). Their use allowed to define Locus Control Regions (LCRs) (270, 271) and important transcription factor binding sites (268, 272).

In order to elucidate regulation of CD69 expression in its genomic environment, we decided to produce BAC in transgenesis of CD69. In this chapter, we first introduce GFP as reporter of CD69 expression in the BACs containing CD69 to detect transgene expression in TG mice or cell lines. Our first purpose was to compare the transgene expression of these BACs with future transgenic expressions of BACs CD69-GFP with deletions in their regulatory regions. For this, we used three different BACs (RP24-109H21, RP24-188C4 & RP24-171I2), containing at least CD69 gene and the four 5' CNSs and spanning more than 50 kb upstream and downstream of the gene and CNSs, to generate BAC-in TG cell lines and BAC-in TG mouse lines. We employ three different BACs in order to discard regulatory effects of isolated fragments that may control other genes not included in the BAC, as the difference of sequences among these three BACs is they cover different ranges of sequences (see Fig. II.M.5, Section II.M).

After BAC modification, we first generated TG mice with GFP/myc reporters inserted in one of the BACs whose CD69 gene & CNSs are located in the center of its genomic sequence (RP24-188C4). As we hypothesized that any non-coding sequence upstream, downstream or within the CD69 gene could regulate

transcription, in order to not affect the BAC CD69 gene expression when generating the CD69 BAC-in reporter, we insert reporters without deleting the CD69 locus sequences. We produce two BAC modifications: *CD69Bi.2* (Fig. II.M.2, Section II.M), which contains an IRES-GFP cassette inserted in the position +40 of the 3' UTR (273); and *CD69Bi.1* (Fig. II.M.2, Section II.M), which is basically the same modification except for containing a *myc* tag inserted just after the Intron V to distinguish transgene from endogenous CD69 expression and from second cistron (GFP) expression. IRES (Internal Ribosomal Entry Sequence) is a DNA fragment vastly employed molecular cloning to allow the transcription of a bicistronic mRNA (273). To bypass the possible problem that bicistronic mRNA instability could affect transgene expression, two *loxP* sites are placed flanking the IRES-GFP sequence in both constructs. If given the case, the IRES-GFP fragment could have been excised by crossing the mice with a Cre-expressing TG line. The expression of GFP and CD69-myc would be detected by flow cytometry directly (the former) and using an anti-myc antibody (the latter).

For the production of TG cell lines, we generated two constructs with GFP fused with CD69 gene in either N-terminal (CD69-NLAP) or C-terminal (CD69-CLAP) ends, and recombined them with the three BACs. Both modifications contain the *neo* gene preceded by an IRES sequence (Fig. II.M.4, Section II.M) in order to select stably transfected clones expressing the transgene. Transfections were performed in a lymphoid cell line (Jurkat) and in a non-hematopoietic cell line (CHO).

Despite using all these different strategies, in contrast to our expectations, BAC transgenesis was unable to generate a model where the BAC-CD69 was regulated similarly to the endogenous CD69. Nevertheless, we found specific patterns of transgene expression in different cell types in the TG mice line with high copy number of the transgene.

MATERIALS & METHODS

BAC modification by homologous recombination

The method used was adapted from (274) and (275). It consists in recombining BAC contained in bacteria with two homologous fragments of DNA previously cloned in a shuttle vector (in our case, between AscI and NotI RE sites) allowing to insert, delete or substitute DNA sequences in the WT BAC.

This shuttle vector contains the *recA, ampR, sacB* genes and R6Kγ ORI (Fig. II.M.1). Recombinase A (produced by *recA* gene) transiently restores in the recombination-deficient host bacteria the ability to catalyze homologous recombination between the BAC and the shuttle vector. The ampicillin resistance gene (*ampR*) permits antibiotic selection different from the BAC selection, which is produced by chloramphenicol resistance gene (*cmR*). The *sacB* gene encodes for the metabolic enzyme *levansucrase*, which transforms sucrose to levan, a toxic product for host DH10B cells in where the BAC is located which allows negative selection of shuttle vector-containing bacteria (276). R6Kγ ORI is an origin of transcription which needs the *Pir* gene to be functional. A *Pir*-dependent origin allows genes located in shuttle vector only be expressed in specific bacterial strains (*Pir2*), or when it has integrated into a BAC in BAC-host cells (DH10B) (277). It has been reported that the *RecA* gene can be expressed in enough amounts to produce homologous recombination even though it is not integrated in the BAC (278) (Fig II.M.1).

The sequence to be recombined with the BAC is contained in a shuttle vector, and is composed at least of 2 DNA boxes of approximately 1 kb each, BOX A and B (Fig II.M.1). These boxes are the responsible for the recombination, and therefore their sequences must be homologous to 2 identical boxes in the BAC sequence. When an insertion in the BAC is desired, in the shuttle vector, A and B need to be separated by the sequence to be inserted (*INS*, Fig II.M.1), and in the BAC, A and B need to be adjacent. To delete a fragment inside BAC (*del*, Fig II.M.1), in the shuttle vector A and B should not have any insert between them, and in the BAC, they have to be separated by the sequence to be deleted. In a substitution, the fragment between A and B in the shuttle (*INS*) replaces the fragment between A and B in the BAC *(del)*. After generating the boxes A and B (and the insert, if applicable) by conventional PCR on genomic DNA template, the joining of boxes A and B (and *INS* in case of Insertion and Substitution) was produced by overlap PCR (216, 274). Then, restriction enzyme (RE) digestion and cloning are performed to generate the shuttle vector.

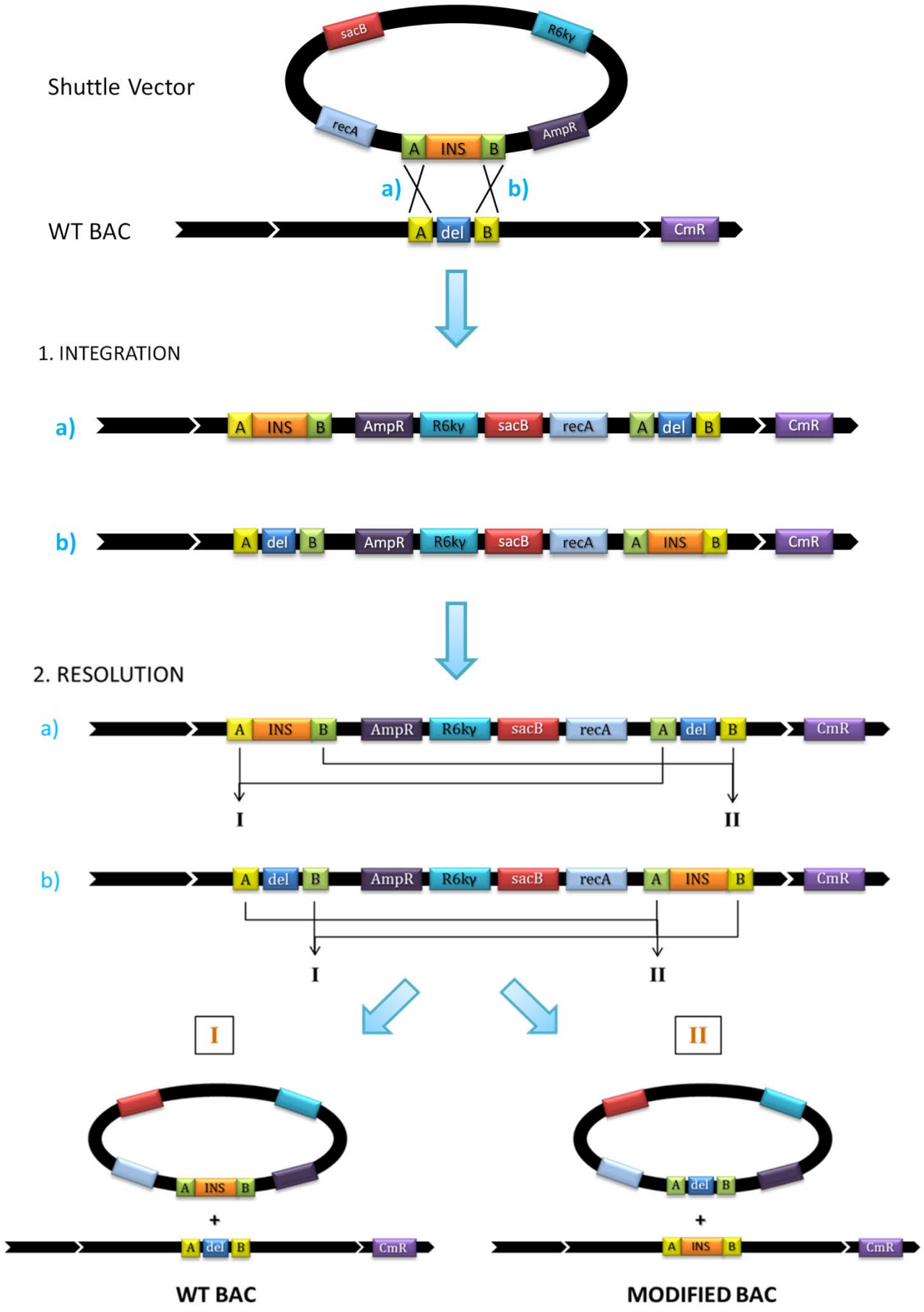

Shuttle Vector
sacB
R6kγ
recA
AmpR
A INS B
a)
b)
WT BAC
A del B
CmR
1. INTEGRATION
a)
A INS B AmpR R6kγ sacB recA A del B CmR
b)
A del B AmpR R6kγ sacB recA A INS B CmR
2. RESOLUTION
a)
A INS B AmpR R6kγ sacB recA A del B CmR
I
II
b)
A del B AmpR R6kγ sacB recA A INS B CmR
I
II
I
II
A INS B
+
A del B CmR
WT BAC
A del B
+
A INS B CmR
MODIFIED BAC

Fig. II.M.1.Modification of Bacterial Artificial Chromosome (BAC) by homologous recombination. The two phases of recombination are represented: **INTEGRATION**, when recombinase A (***recA***) from shuttle vector inserts the shuttle vector sequence into unmodified BAC (***WT BAC***) by recombining box A (depicted as ***a)***) or box B (depicted as ***b)***);. and phase 2, **RESOLUTION**, both insertions have 2 possibilities of recombination, depending on if they recombine box A or box B: **I)** BAC excises the original shuttle vector and remains intact; **II)** Recombination occurs in the box that did not recombined in phase 1, and integration of insert, deletion or substitution is produced in the BAC. For insertion, *del* sequence is absent and A and B in the original BAC are adjacent; for deletion, *INS* is absent and A and B are adjacent in the shuttle vector; and for substitution, both *INS* and *del* are present. ***R6kγ***, R6K plasmid origin of replication; ***AmpR***, ampicillin resistance gene; ***CmR***; chloramphenicol resistance gene; ***sacB***, levansucrase gene; ***INS***, sequence to insert in the WT BAC; ***del***, sequence to delete of the WT BAC.

Recombination between BAC and the shuttle vector was performed by electroporation of a concentrated aqueous solution of the shuttle vector into electro-competent bacterial cells containing the target BAC. The molecular process occurs in 2 steps (Fig. II.M.1):

a) Integration: BOX A or BOX B of the shuttle recombines with its homologous sequence in the BAC. This produces the insertion of the complete vector between BOX A and BOX B of the BAC. Depending on what box recombines first, two different insertions can be produced (Fig. II.M.1-1).

b) Resolution: A second recombination occurs and 2 results may be obtained: Successful recombination, where the insert DNA (*INS*) remains in the BAC (insertion) or the DNA target to be deleted from the BAC (*del*) is taken by the shuttle (deletion), or both, in case of substitution (Fig. II.M.1-2.II); the other result is obtaining the initial BACs and shuttles (Fig. II.M.1-2.I).

To isolate single colonies of the Integration phase, double selection of antibiotic is employed in plates and growth media, as the BAC with the integrated shuttle carries resistance to Ampicillin in addition to Chorampenicol resistance characteristic of WT BAC. Not integrated shuttle vectors cannot confer resistance to Ampicillin as R6Kγ origin does not work in BAC host cells. To select positive clones in both steps it is necessary to screen the surviving clones by PCRs. Positive colonies are then plated on minimal media plates containing only sucrose, in order to activate the *sacB* gene of the shuttle vector. The colonies with shuttle vector integrated and not resolved would not survive in minimal media, as *sacB* protein would be expressed and would produce a toxic compound for the bacteria (274).

Finally, the positive colonies are amplified and purified for subsequent applications.

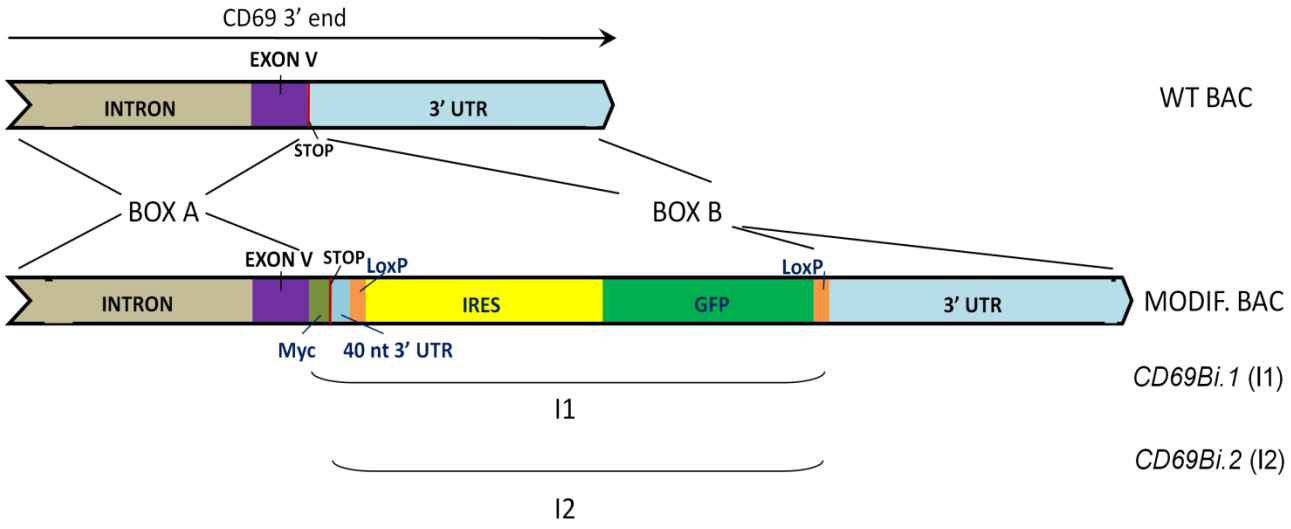


Fig. II.M.2. CD69-WT BAC modification for generation of transgenic mice lines CD69Bi.1 and CD69Bi2. *WT BAC*: Sequence of 3' end of CD69 gene in non-modified BAC. *MODIF. BAC*: Modifications introduced in CD69 gene of WT BAC; for *CD69.Bi.1*, *myc* epitope, CD69 STOP codon plus the next 40 nt of 3' UTR, LoxP sequence in 5', IRES sequence, GFP gene, LoxP 3' sequence **(I1)**; for *CD69.Bi.2*, CD69 STOP codon, 5' LoxP, IRES, GFP and 3' LoxP **(I2).**

❖ Shuttle Construction

- FOR THE GENERATION OF TRANSGENIC MOUSE LINES:

CD69Bi.1: As the recombination final effect is an insertion, the insert of shuttle to clone was: BOX A + I1 (that contains the myc tag and the GFP cDNA preceded by an IRES sequence)+ BOX B (Fig. II.M.2). I1 construct was obtained from 3 steps of subcloning in pMIG vector (279). To assemble the A + I1 + B, an overlap PCR (Fig. II.M.3) was set up with the primers in Table II.M.I.

CD69Bi.2: For BOX A + I2 (contains GFP cDNA preceded by an IRES sequence) + BOX B insertion, the same procedure than with *CD69Bi.1* was followed (Fig. II.M.2). Primers for overlap PCR are displayed in Table II.M.I.

- FOR THE GENERATION OF TRANSGENIC MOUSE CELL LINES:

Constructs to modify BACs for transfection in cell-lines were obtained from Dr. Frank Buchholz (262). These constructs are:

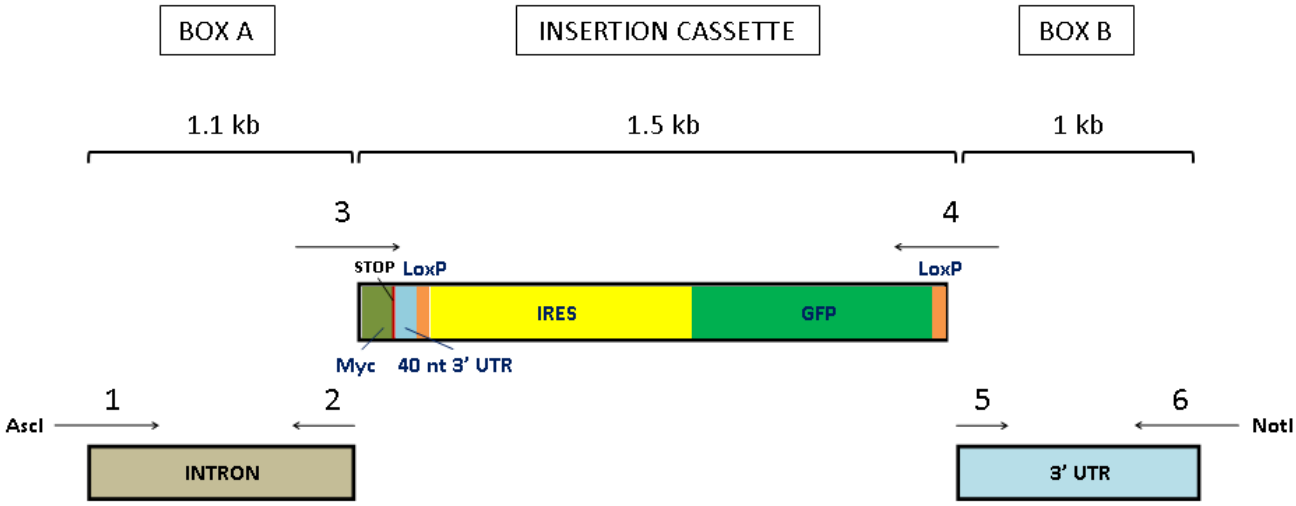


Fig. II.M.3. Overlap-PCR scheme for insertion of *I1* in shuttle vector. In an initial step the 3 individual fragments are amplified in single PCRs (BOX A, I1, BOX B). These 3 isolated and purified fragments were used as template for a second PCR using primers 1 and 6. The resulting 3,6 kb fragment was cloned into shuttle vector (AscI and NotI RE sites) and confirmed by sequencing.

CD69-NLAP: N-LAP tag contains an e-GFP gene split in 2 exons by a sequence with the kanamycine/neomycine resistance gene directed by 2 promoters: *PGK* promoter, for eukaryotic expression, and *gb2* promoter, to direct expression in bacteria (Fig II.M.4). The intron disrupting GFP is flanked by splicing signals to allow its excision during transcription in mammal cell cultures.Overlap PCR was employed in 2 steps to clone the constructs: first, joining of N-LAP, previously amplified in 3 sequences; second, amplification of the whole insert BOX A - NLAP - BOX B. BOX A and B were approximately 1 kb long: "A" sequence contains DNA upstream *position +1* (promoter + 5' UTR); "B" sequence starts at *+1*.

CD69-CLAP: genes in this construct are eGFP and *neoR*, the latter preceded by an IRES sequence to allow the bicistronic transcription. The aim was to insert the tag after the 5th exon, being eGFP in frame with mCD69 mRNA, and before 3' UTR. BOX A and BOX B expanded 1 kb upstream and downstream respectively from the insertion position. Overlap PCR was performed in 2 steps as for CD69-NLAP: first, joining of C-LAP from 3 fragments and after that, synthesizing the complete insert.

Primers containing the RE sites AscI and NotI used in both constructs are displayed in Table II.M.II. Integrity of all shuttle constructions sequences was checked by sequencing.

Table II.M.I. Primers designed for generation of BAC-in transgenic mice lines.

Name	Sequence 5' ---> 3'
pMIG construction	
LoxP1_F	AATTCGTTAACCTCGAGATAACTTCGTATAGCATACATTATACGAAGTTATG
LoxP1_R	TCGACATAACTTCGTATAATGTATGCTATACGAAGTTATCTCGAGGTTAACG
LoxP2_F	GGCCACATAACTTCGTATAGCATACATTATACGAAGTTATG
LoxP2_R	TCGACATAACTTCGTATAATGTATGCTATACGAAGTTATGT
Myc_F	GTCTGCAGCAAGCCCTCCAGAGCAGAACAAAAACTCATCTCAGAAGAGGATCTGAATGGGGCCGCATGACGAGGATACATAGATGTAT
Myc_R	ATACATCTATGTATCCTCGTCATGCGGCCCCATTCAGATCCTCTTCTGAGATGAGTTTTTGTTCTGCTCTGGAGGGCTTGCTGCAGAC
Shuttle modification	
BAC.1	TAGGCGCGCCAGTAACTCCGTCGCATCACTTAAC
BAC.2	TCTGGAGGGCTTGCTGCAGACCCA
BAC.3	TGGGTCTGCAGCAAGCCCTCCAGATGACGAGGATACATAGATGTATAA
BAC.3_G *	TGGGTCTGCAGCAAGCCCTCCAGATGACGAGGATACATAGATGTATAA
BAC.4	GAGCAGTATCCAGTTACACAACTTTATAACTTCGTATAATGTATGC
BAC.5	AAAGTTGTGTAACTGGATACTGCTC
BAC.6	ATGCGGCCGCCAAAGACTCTTCTCTGGTGAGGTT
Screening	
recA_1F	ACACAGCTCCCATCACTGCT
recA_2R	CCCTAGGAATGCTCGTCAAG
recB_3F	CGACCACTACCAGCAGAACA
recB_4R	CTGTGCAGGTCTTGTGGCTA
Genotyping	
GFP_F	CACGACTTCTTCAAGTCCGC
GFP_R	CTCCAGCAGGACCATGTGAT
IRES_F	CGAAGCCGCTTGGAATAAGG
IRES_R	ACCTTCTGGGCATCCTTCAG
5END_F	CGGTCGAGCTTGACATTGTA
5END_R	GTTCCCATCAGCAATGGAGT
3END_F	CCAAGGGCAAGTTCATCATT
3END_R	GCTGCTGTTTAGGGATCTGC
Real-time PCR	
RT_IRES_F	CAAATGGCTCTCCTCAAGCG
RT_IRES_R	ACCTTCTGGGCATCCTTCAG
RT_Jun_F	CCCTGCATGCTATCATTGGC
RT_Jun_R	GTCTGGACTTGTGTGTTGCC

* For CD69Bi.1 and CD69Bi2 same primers for shuttle construction were employed, except for BAC.3_G, which was used for generation of CD69Bi2, instead of BAC.3 (used for CD69Bi.1).

❖ BAC recombination

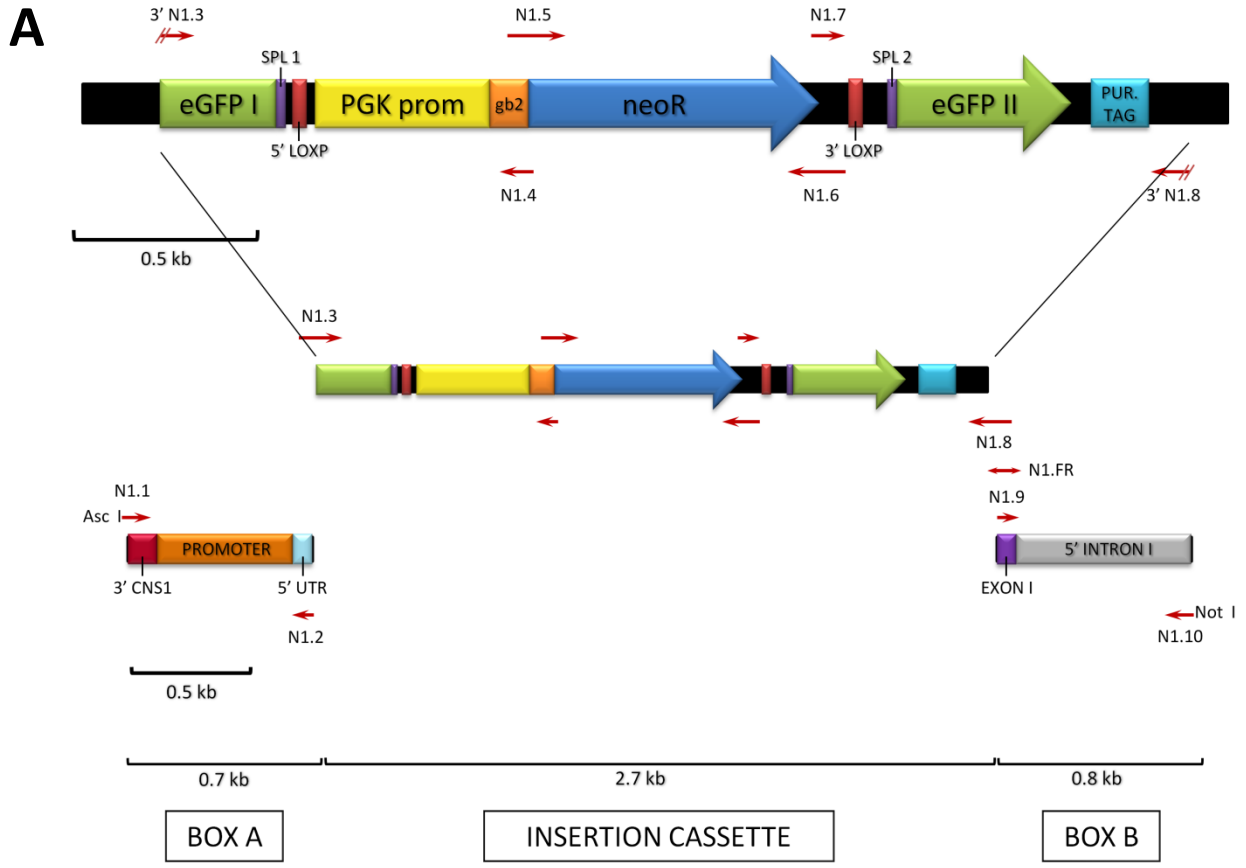
A wide collection of genomic libraries are available at the *BACPAC Resource Center* (BPRC) bank (http://bacpac.chori.org/home.htm), including mice and human genomic clones. Several BACs from murine RP23 and RP24 libraries contain the CD69 gene and the 4 CNSs upstream of it. The vector base of BAC RP24-male mouse library is *pTARBAC1* (247), which allows to clone BAC in yeast, in contrast with RP23, a female mouse library based on pBACe3.6 "classic" vector (280). Bacterial clones of three RP24 BACs containing the CD69 gene were purchased for modification: RP24-109H21, RP24-188C4 & RP24-171I2 (Fig. II.M.5). They contain an inserted genomic sequence of 177, 165 and 166 kb respectively.

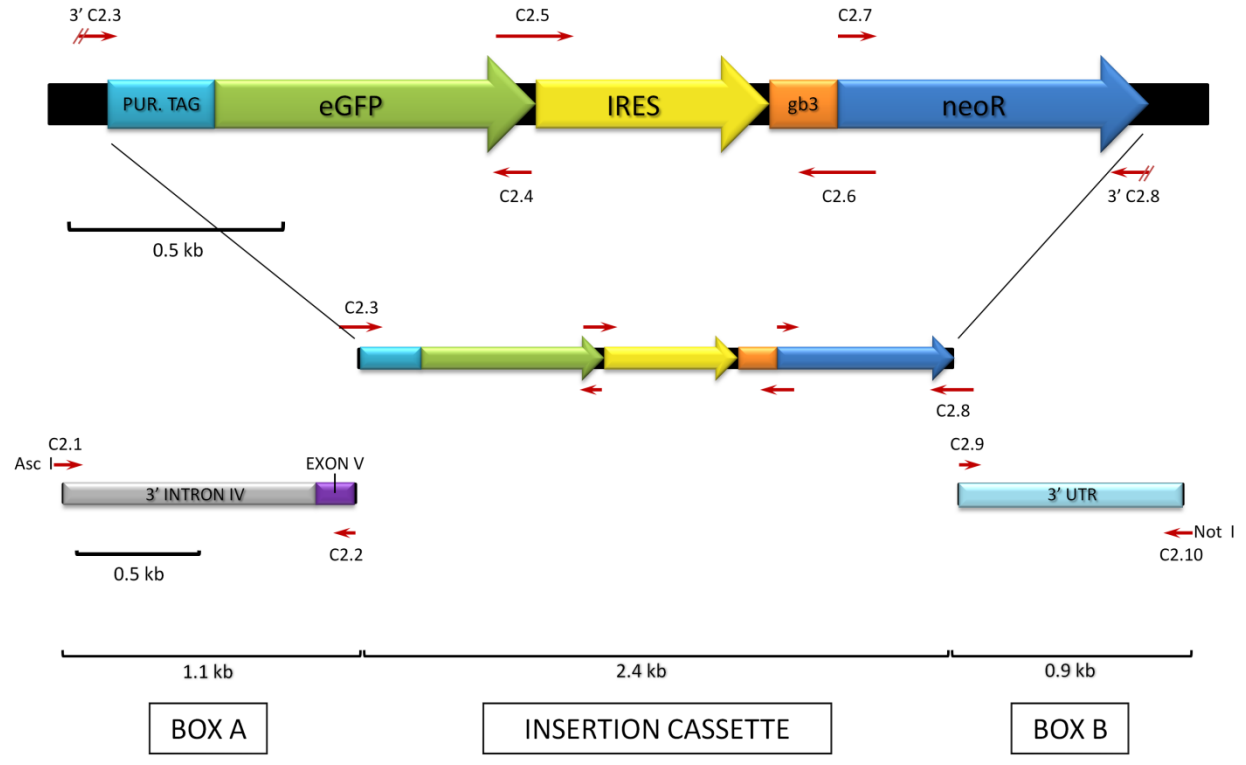
The first step was to produce electrocompetent cells from BAC-containing bacteria (pellet of 250 ml of overnight-growth culture) by washing them 3 times with cold 10% glycerol and resuspending them in 1 ml 10 % glycerol. After that, 30-50 µl of electrocompetent bacteria were electroporated with 500 ng to 1 µg of shuttle vector maxiprep solution in 1 mm-gap cuvettes (BTX, Harvard Instruments Inc.) employing a *Bio-Rad GenePulser* (Bio-Rad) and the settings 1800 V, 25 µF, 200 Ω. Cuvette content was collected with 500 µl to 1 ml of SOC medium and grown at 37º C, 220 rpm for 1 hour.

For selection, bacteria were plated in Lysogeny broth (LB)-agar plates supplemented with Ampicillin (50 µg/ml) and Chloramphenicol (12.5 µg/ml) and grown overnight at 37º C. Next day some colonies were picked and re-suspended in 30 µl of LB medium to use them as DNA template in the first screening PCR (for primers employed in different constructs, see Tables II.M.I and II.M.II).

Colonies that had integrated the shuttle vector were grown overnight at 37º C, 220 rpm in LB medium with 50 µg/ml Ampicillin and 12.5 µg/ml Chloramphenicol and plated in M9 minimal media plates ((281), with modifications).

Fig. II.M.4. Overlap PCR scheme for shuttle vector construction for *CD69-NLAP* and *CD69-CLAP* BAC modifications. *A)* Synthesis of insert for CD69-NLAP modification: Insertion cassette was amplified in 3 steps by overlap PCR (primers employed: N1.3, N1.4, N1.5, N1.6 N1.7, N1.8 and DNA fragment N1.FR) and then joined to box A and B by a second overlap PCR (using N1.1 and N1.10 as primers). *B)* Insert for CD69-CLAP modification: Insertion cassette was amplified in 3 steps by overlap PCR (primers employed: C2.3, C2.4, C2.5, C2.6 C2.7 and C2.8) and then joined to box A and B by a second overlap PCR (using C2.1 and C2.10 as primers). **eGFP**, Green Fluorescent Protein cDNA; **eGFP I** and **eGFP II**, eGFP cDNA excised in 2 exons; **IRES**, Internal Ribosome Entry Site; **neoR**, neomycin/kanamycin resistance gene; **PUR. TAG**, purification tag to allow the isolation and purification of the resulting protein; **SPL**, splicing sites; **loxP**, recombination sites for Cre recombinase; **PGK prom**, eukaryotic PGK promoter; **gb2** and **gb3**, bacterial promoters; **3' CNS1**, 3' region of Conserved Non-coding Sequence 1 of mCD69; **PROMOTER**, promoter of mCD69; **5' UTR**, 5' Un-Translated region of mCD69; **EXON I**, complete sequence of mCD69 exon I; **5' INTRON I**, 5' end of mCD69 intron I; **3' INTRON IV**, 3' sequence of mCD69 intron IV; **EXON V**, complete sequence of exon V in mCD69; **3' UTR**, complete sequence of 3' Untranslated Region of mCD69.


A
3' N1.3
N1.5
N1.7
SPL 1
SPL 2
eGFP I
PGK prom
gb2
neoR
eGFP II
PUR. TAG
5' LOXP
3' LOXP
N1.4
N1.6
3' N1.8
0.5 kb
N1.3
N1.8
N1.FR
N1.1
Asc I
N1.9
PROMOTER
5' INTRON I
3' CNS1
5' UTR
EXON I
Not I
N1.2
N1.10
0.5 kb
0.7 kb
2.7 kb
0.8 kb
BOX A
INSERTION CASSETTE
BOX B


B
3' C2.3
C2.5
C2.7
PUR. TAG
eGFP
IRES
gb3
neoR
C2.4
C2.6
3' C2.8
0.5 kb
C2.3
C2.8
C2.1
Asc I
EXON V
C2.9
3' INTRON IV
3' UTR
Not I
C2.2
C2.10
0.5 kb
1.1 kb
2.4 kb
0.9 kb
BOX A
INSERTION CASSETTE
BOX B

After 2-3 days grown colonies were screened for resolution with primers in Tables II.M.I and II.M.II.

Generation of transgenic mice

Generation of mouse TG lines was produced by pronuclear microinjection of the BAC into mouse oocytes. Its purity and integrity are critical for transgenesis efficiency (282).

Bacteria containing modified BAC were grown overnight in LB medium with 12.5 μg/ml Chloramphenicol and the BAC purified with *QIAGEN Large-Construct Kit* from QIAGEN and re-suspended in polyamine (PA) buffer which was pre-filtered through Anotop inorganic membrane filters (average pore size: 0.02 μm from Whatman, Maidstone, England). PA buffer (10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 30 μM spermine, 70 μM spermidine, 100 mM NaCl) was prepared as described (283).

BAC integrity was evaluated by pulsed-field gel electrophoresis (PFGE) and diluted in PA buffer to a suitable concentration for successful microinjection.

Concentrations of BAC of 0.5 and 1 ng/ul were microinjected into CBA mouse strain oocytes at the Animal Facility of CNB (Centro Nacional de Biotecnología, Madrid, Spain) as described (282). Positive founders were crossed with C57BL/6 individuals for at least 4 generations, genotyped and sacrificed for expression analyses by flow cytometry.

Genotyping

Mouse tails were collected and DNA was extracted. First, tails were digested in 500 μl of SNET Buffer (10mM Tris pH 8.0, 0.1M EDTA, 0.5% SDS) (281) supplemented with 400 μg/ml of proteinase K and heated overnight at 56 ºC. The next day samples were centrifuged to eliminate solid undigested material and the DNA in the supernatants was extracted with 500 μl of phenol - phenol:chloroform (1:1) and chloroform. DNA was precipitated from aqueous solution adding 500 μl isopropanol , washed with 200 μl of 70% Ethanol and resuspended in 50 - 100 μl of TE_{10} buffer. (10mM Tris-HCl pH 8, 0.1mM EDTA)

Primers for routine genotyping PCR (*AmpliTaq* from Applied Biosystems) were designed to amplify a fragment of eGFP, for a fragment of IRES, and for both junctions of BAC genomic insert with BAC backbone (Table II.M.I).

Table II.M.II. Primers designed for generation of BAC-in transgenic Jurkat cell-lines.

Name	Sequence 5' ---> 3'
Shuttle Construction: CLAP	
C2.1	TAGGCGCGCCAGTAACTCCGTCGCATCACTTAAC
C2.2	TCTGGAGGGCTTGCTGCAGACCCA
C2.3	TGGGTCTGCAGCAAGCCCTCCAGAGATTATGATATTCCAACTACTG
C2.4	AGATCATTACTTGTACAGCTCGTC
C2.5	GACGAGCTGTACAAGTAATGATCTAGAGTCGAGTTAATTAAGAA
C2.6	TCTTGTTCAATGGCCGATCCCATAACACCCCTTGTATTACTGT
C2.7	ATGGGATCGGCCATTGAACAAGA
C2.8	TCATTTATACATCTATGTATCCTCGTCAGAAGAACTCGTCAAGAAGG
C2.9	TACATAGATGTATAAATGACTGTGCCATA
C2.10	ATAAGAATGCGGCCGCGGACTGGGAAAGACACAAACA
Shuttle Construction: NLAP	
N1.1	TTGGCGCGCCGCACTAAAGCAACTCCTGACACT
N1.2	TTTTCCCCTCAAGGTCCCTGGCGACTC
N1.3	GAGGGGAAAAAAATTAAAAAGGATGGTGTCCAAGGGCGAGGA
N1.4	TTTAGTTCCTCACCTTGTCGTATT
N1.5	AATACGACAAGGTGAGGAACTAAACCATGGGATCGGCCATTGAAC
N1.6	ATTCGCCAGGGAGCTCTCAGACGTGAATAAAGACCGACCAAGCG
N1.7	ACGTCTGAGAGCTCCCTGGCGAAT
N1.8	ATAGAACAGTTTTCAGAATCCCCTGGGCAGGTCGTCGGTCA
N1.9	CTATAACGGAAAATAGCTCTTCACATC
N1.10	ATATGACAGCGGCCGCATAACTCTCATTCCCAAGTGCTAAGT
N1.FR_S	GATTCTGAAAACTGTTCTATAACGGAAAATAGCTCTTCACATC
N1.FR_AS	GATGTGAAGAGCTATTTTCCGTTATAGAACAGTTTTCAGAATC
Screening	
REC_N1BOXA_scr_1F	CAAGAAAATGAGCAAGGGATG
REC_N1BOXA_scr_1R	GCCGTAGGTCAGGGTGGT
REC_N1BOXB_scr_1F	GAGGAGGAGGCGAGAACC
REC_N1BOXB_scr_1R	GGTCTTTCCTTCATTCCACTATTT
REC_C2BOXA_scr_1F	TGGTTCAGTTCTTCCTCACACA
REC_C2BOXA_scr_1R	AACCTCCACCTCCGCTGT
REC_C2BOXB_scr_1F	CTACCCGTGATATTGCTGAAGAG
REC_C2BOXB_scr_1R	GAATGAGAACCGCTGGAAAC

Analysis of transgene copy number in CD69-GFP in transgenic mice

DNA previously extracted from tails of 3 mice positive for GFP genotyping (of each line) was further purified with silica columns, diluted and analyzed by Real-Time PCR. Real-time was performed using *LightCycler® FastStart DNA Master^{PLUS} SYBR Green I* from Roche, using primers for a 54 nt-amplicon of IRES (primers in Table II.M.I).

The standard curve was done with a mixture of genomic DNA from non-TG mice and purified BAC previously used to microinject the *CD69Bi.2* line. Previously, several dilutions of genomic DNA and BAC were tested separately, resulting in equivalent fluorescence signal for 1 pg of genomic DNA (*c-Jun,* reference gene) and 0.2 pg of BAC (IRES). As 1 "copy" of genome contains 2 copies of *c-Jun*, we established a ratio 10:1 (weight) of genome:BAC to 1:1 (copies). To generate the standard curve, a total of 20 ng of DNA was mixed with several quantities of BAC, at 100000:1, 40000:1, 20000:1 and 10000:1 genome:BAC proportions (weight), and the normalized results obtained from *Light Cycler Software* were multiplied by 10^3.

BAC Transfection in Jurkat cell line

Previously to the transfection, a kill curve of G418 for Jurkat was needed. We chose at which cells started dying started at 5 days and were completely dead at 10 - 12 days. The concentration of selection of G418 for Jurkat was established to be 600 µg/ml.

BACs were isolated and purified with *Plasmid Maxi Kit* from QIAGEN and dissolved in TE_{10} buffer (10mM Tris-HCl pH 8, 0.1mM EDTA).

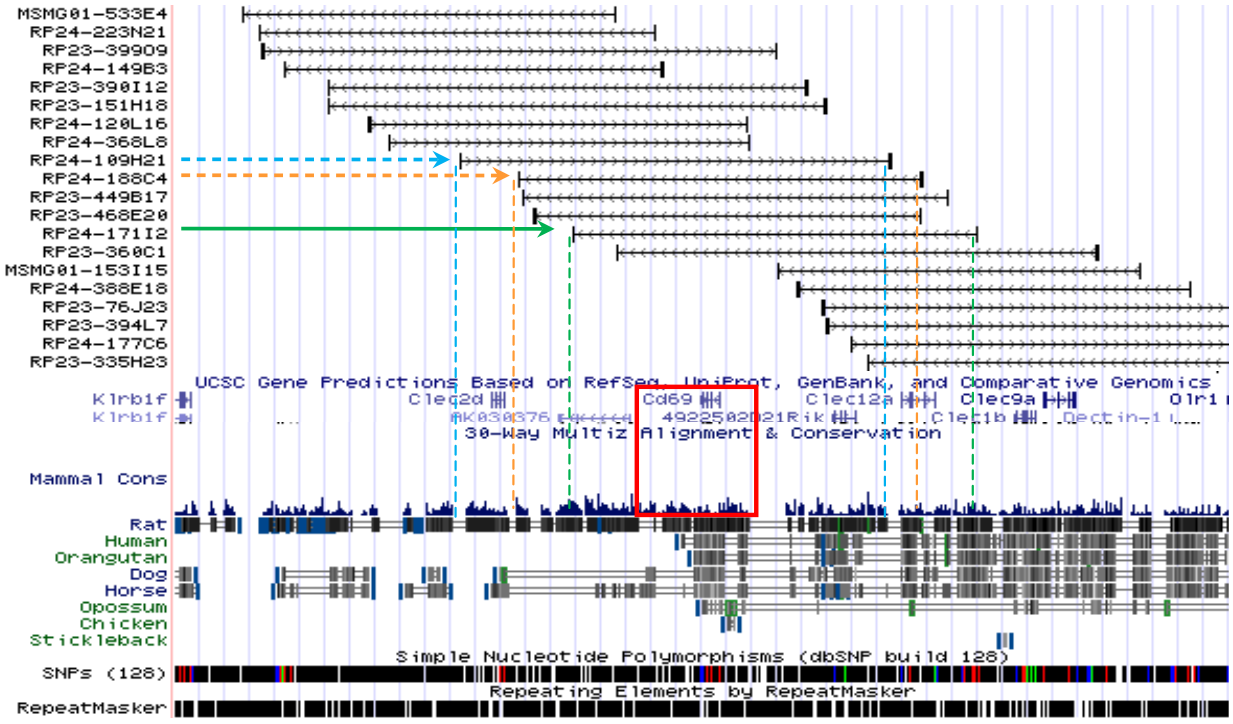


Fig. II.M.5. mCD69 BAC library clones selection. VISTA tracks showing BAC mouse library clones (top) aligned with mouse sequence and mammal conservation (mid). Bottom, *Single Nucleotide Polymorphisms* (SNPs) and *Repeating Elements*. Coloured arrows indicate BAC constructs selected. Red square, CD69 gene with 5' CNSs.

Before transfection of CD69-BAC constructs into Jurkat, BAC-transfection was tested employing two BACs: 3276 (mSFRS4-LAP) and 3715 (mH2AF7-LAP), which were kindly provided by Dr. Frank Buchholz (262). Jurkat cells were plated at 3-5 million cells per well in 6-well plates and were transfected with *Superfect* (Invitrogen) and different quantities of BAC, according to manufacturer's instructions. We tested 0.25, 0.50 and 1 μg of BAC per point with 4 and 8 ul of transfection reagent for both BACs.

After 24 h, cells were washed with 1x PBS and resuspended in fresh complete medium. The next day, medium was changed again and supplemented with 600 μg/ml of G418 selection reagent. During the following 2 weeks, media with G418 was renewed every 2 - 3 days. After 12-15 days, all cells transfected with the negative control BAC (RP24-188C4ΔsacB) had died, and 5-15 clones survived in the control positive wells. The best percentage of survival to G418 was obtained in the transfections done with 500 μg of BAC and 8 μl of transfection reagent. Positive clones that had survived in this condition were harvested, expanded and analyzed by flow cytometry.

Cell samples preparation for flow cytometry

6 - 12 weeks old mice were sacrificed and several tissues and fluids (spleen, thymus, blood, lymph nodes (LN)- brachial, axillary, inguinal popliteal and maxilary- and bone marrow (BM)) were collected to obtain cells of specific lineages of interest. Tissues were mechanically disaggregated and cells were harvested, washed twice with 1x PBS and pelleted. Blood samples (diluted at extraction at least 10 times in 1x PBS to avoid coagulation) were centrifuged and supernatant removed. After that, all samples, except for LN samples, were lysed with Lysis Buffer (0.15 M NH4Cl, 1mM KHCO3, 1mM Na2-EDTA, pH 7.4) for a maximum of 2 min. Samples were washed twice with 1x PBS and pelleted for immunostaining.

Cultured human cells lines were washed twice in 1x PBS prior to stimulation and/or staining.

When stimulation was performed (only for splenocytes and LN cells), cells were cultured in complete media for 6 hours at 37 ºC, 5% CO_2. 10 ng/ml of PMA and 500 ng/ml of Ionomicin were added at different times to establish a time-course activation curve. Then they were washed in twice in 1x PBS and pelleted for staining and flow cytometry analyses.

Flow cytometry analyses

Mouse or human cells were immunostained for analysis by Flow Cytometry. Purified rat anti-mouse CD16/CD32 (*Fc Block* from Becton-Dickinson) was previously added to cell samples to avoid unspecific binding of antibodies to Fc receptors. Staining was done for 20 min at 4º C with FITC-, PE- , APC-, PeCy5- and/ or PECy7- conjugated antibodies or biotinilated antibodies, all diluted in staining buffer

Table II.M.III. Antibodies employed in Flow Cytometry analyses of transgenic tissues.

Target	Fluorochrome[3)]	Clone	Supplier	Cat No.
B220/mCD45R	(biotin)[4)]	RA3-6B2	eBioscience	13-0452
mCD11b	(biotin)[4)]	M1/70	BD Pharmingen	553309
mCD44	(biotin)[4)]	IM7	eBioscience	13-0441
mGr-1[1)]	(biotin)[4)]	RB6-8C5	BD Pharmingen	553124
mNK1.1	(biotin)[4)]	PK136	Immunotools	22155251
mCD11c	PE	N418	eBioscience	12-0114
mCD19	PE	6D5	Immunotools	22190194
mCD49b/DX5	PE	DX5	BD Pharmingen	553858
mCD62L	PE	MEL-14	Immunotools	22159624
mCD69	PE	H1.2F3	eBioscience	12-0691
mCD117/c-kit	PE	2B8	eBioscience	12-1171
mFoxP3	PE	FJK-16s	eBioscience	12-5773
SAV[2)]	PE	-	eBioscience	12-4317
SAV[2)]	PE	-	BD Pharmingen	554061
B220/mCD45R	APC	RA3-6B2	eBioscience	17-0452
mCD8a/Ly-2	APC	53-6.7	eBioscience	17-0081
mCD25	APC	PC61.5	eBioscience	17-0251
mCD117/c-kit	APC	2B8	eBioscience	17-1171
mGr-1[1)]	APC	RB6-8C5	eBioscience	17-5931
mIgM	APC	II/41	eBioscience	17-5790
SAV[2)]	APC	-	eBioscience	17-4317
mCD4	PE-Cy7	RM4-5	eBioscience	25-0042
mCD69	PE-Cy7	H1.2F3	eBioscience	25-0691
SAV[2)]	PE-Cy7	-	eBioscience	25-4317
mCD4	PerCP	RM4-5	BD Pharmingen	553052
hCD69	PE-Cy7	FN50	eBioscience	25-0699

[1)] Common epitope from mLy-6G, mLy-6C.

[2)] Streptavidin-fluorochrome conjugates.

[3)] Fluorochromes: PE: Phycoerythrin; FITC: fluorescein isothiocyanate; APC: Allophycocyanin; PE-Cy7: conjugate system that combines Phycoerythrin and a cyanine dye (Cy7).

[4)] Biotin-conjugated primary antibodies, they need a staining with *Streptavidin* conjugated with a fluorochrome (i. e. SAV-APC).

(1x PBS supplemented with 2% of Fetal Bovine Serum and 2mM of EDTA) (the complete list of antibodies is shown in Table II.M.III). In samples stained with biotinilated antibodies a second staining was performed with streptavidin- fluorochrome conjugates (Table II.M.III). After primary and secondary staining, cells were washed twice with staining buffer. 7-AAD (BD Pharmingen) was added after washing to exclude dead cells.

Samples were analyzed with flow cytometers *FACSCanto* and *FACSCalibur* (Becton Dickinson) and the software employed to analyze the data was *FACSDiva* (Becton Dickinson) and *FlowJo* (TreeStar Inc.).

RESULTS

Generation of Bac-in transgenic mice lines

Our main objective was to study the CD69 regulation in its genomic context to allow all the endogenous regulation mechanisms to function, and therefore to be evidenced. Bacterial Artificial Chromosomes (BACs) are characterized by the capacity to contain intact 100 -200 kb fragments, that allow inserting a whole gene with its distal regulatory regions into genomic chromosomes by BAC transgenesis, that is, a transgene that mimics the endogenous locus.

The modified BAC *CD69Bi.1*, containing GFP and *myc* tag as reporters, was microinjected to generate the transgenic mice line *CD69Bi.1,* obtaining 5 founders (positive for GFP PCR amplicon) from 34 individuals. The modified BAC *CD69Bi.2*, with only GFP as reporter, was used to obtain the *CD69Bi.2* transgenic line finding 4 founders from 37 screened individuals.

All the 9 founders were crossed with C57BL/6, being 7 of the 9 fertile, and 4 of the fertile had positive offsprings (positive for GFP PCR amplicon). Remarkably, those 4 fertile founders were male. Percentage of positive offspring was variable among lines, being the least frequent in the *CD69Bi.1.26* transgenic line (7%) and the most frequent in the *CD69Bi.2.8* line (62%). Mice from the 4 lines developed normally and showed no apparent defects.

Tail DNA from founders was checked for the presence of IRES, GFP and both junctions between the end of the BAC genomic DNA and the BAC backbone to study the integrity of the BAC inserted in the endogenous genomic DNA (Table II.R.I). 6 of the 9 founders had the complete insert. The PCR signal of at least one of the 4 amplicons of the other 3 founder lines (*CD69Bi.1.7*, *CD69Bi.2.7* and *CD69Bi.2.34*) were negative or weak respect to the equivalent signal of the other founders.

The number of copies of BAC-insert in transgenic lines was analyzed by Real-Time PCR (Figure II.R.1). All mouse lines were found to carry low-copy number of transgene except *CD69Bi.1.26*, which carried an average number of 32 transgene copies. This high-copy number of transgene may have produced an insertion in tandem into genomic DNA of more than 5 Mb, a putative explanation for the low transgene transmission of this line.

Table II.R.I. Analysis of CD69Bi.1 and CD69Bi.2 transgenic mouse lines.

TRANSGENIC LINE	Insert Integrity	Fertile (Y/N)	Positive Offspring (Y/N) (%)	GFP expression (Y/N)
CD69Bi.1				
♂ **1.7**	NO 3' NO IRES	Y	Y (48%)	N
♀ **1.17**	COMPLETE*	Y	N	-
♂ **1.26**	COMPLETE*	Y	Y (7%)	Y
♀ **1.30**	COMPLETE*	N	-	-
CD69Bi.2				
♀ **2.3**	COMPLETE*	Y	N	-
♂ **2.7**	NO 5'	Y	Y (25%)	N
♂ **2.8**	COMPLETE*	Y	Y (62%)	N
♀**2.20**	COMPLETE*	Y	N	-
♀**2.34**	NO 3'	N	-	-
TOTAL	6/9	7/9	5/7	1/4

*"COMPLETE" is considered when 5', 3' ends, IRES and GFP PCRs are all positive in this TG line.

BAC-in CD69-GFP expression is abrogated in low copy transgenic mice

The four positive fertile founder mice produced the corresponding TG lines, which were analyzed for expression of GFP by flow cytometry. We found that GFP was only detected in the TG line with the highest copy number of BAC transgene, *CD69Bi.1.26* (from now on, named as *CD69.BAC*). The three mouse lines containing low copy number of the CD69-BAC reporter (*CD69Bi.1.7*, *CD69Bi.2.7*, and *CD69Bi.2.8*) did not express GFP in spleen or thymus, even under stimulatory conditions, despite expressing normal levels of CD69 (Fig. II.R.2). Conversely, the *CD69.BAC* TG line, which contains ~32 copies of the CD69-BAC construct, show mCD69 expression in all hematopoietic tissues studied, but with different patterns of expression (Fig. II.R.3).

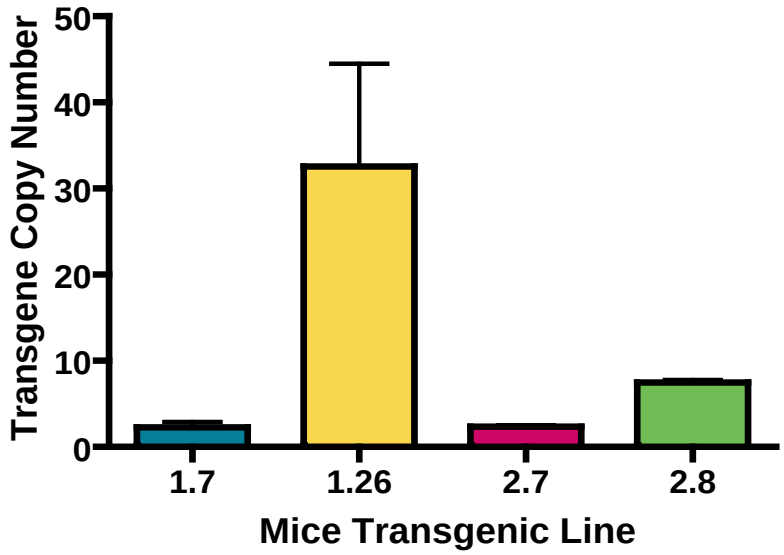


Fig. II.R.1. Number of BAC copies integrated in CD69-BACin mouse transgenic lines. Number of copies was determined by amplifying a 54 bp-sequence of IRES and calculated by relative quantification. The reference gene used was *c-Jun*.

CD69-BAC transgene is differentially regulated among tissues and cell-types in vivo

Spleen, thymus, lymph nodes, blood and bone marrow of *CD69.BAC* mice were analyzed by flow cytometry, and we observed that, this high expression of the transgene varies among tissues in a specific manner. In the bone marrow, hematopoietic precursors c-kit+ (CD117+) (Fig II.R.4A) as well as immature and mature B cells (CD19+) and granulocytes (Gr-1+) (Fig. II.R.4B & II.R.4C, respectively), expressed CD69 and GFP at intermediate levels in TG mice.

In this tissue there is a small population of non-expressing-transgene cells (also non-expressing endogenous CD69), which do not correspond to any of the previous populations nor to T cells (CD3+), natural killer (NK) cells (DX5+) or mature conventional dendritic cells (cDCs) ($CD11c^{high}$). We hypothesized that at least some of these cells are very early progenitors, as pluripotent hematopoietic stem cells are c-kit- (284). When comparing c-kit vs. GFP, CD69 vs. c-kit and CD69 vs. GFP graphs, a gene expression sequence of endogenous and transgenic CD69 could be inferred if it is assumed that c-kit expression precedes the one of CD69 or GFP: 1) c-kit expression occurs and increases to high levels before CD69 expression (orange population depicted in Fig. II.R.5A), 2) the transgene becomes expressed and GFP expression peaks (red and light green populations, Fig. II.R.5b), 3) as maturation progresses, c-kit is slightly down-regulated together with GFP and CD69 (cells in dark green, Fig. II.R.5C).

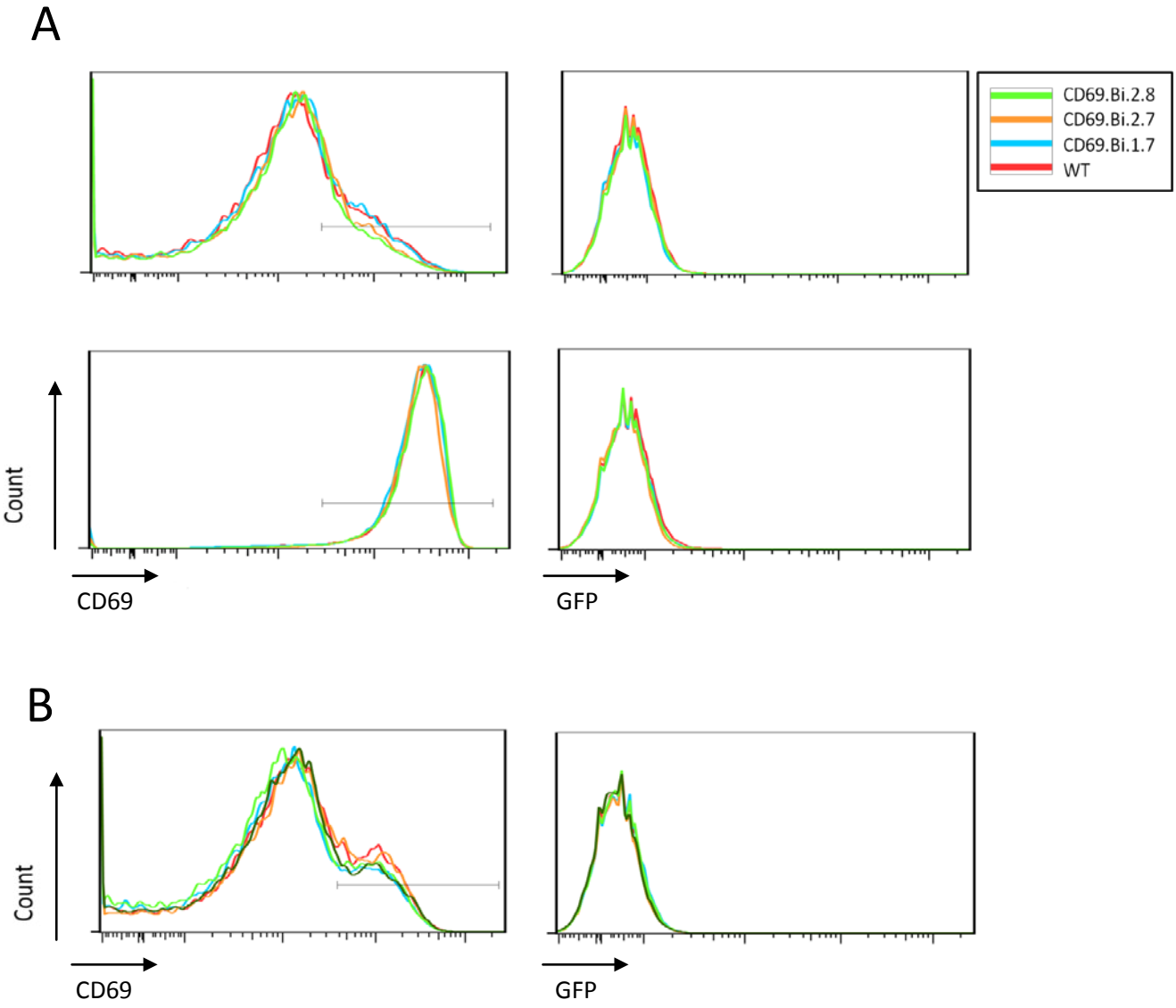


Fig. II.R.2. No transgene expression in CD69Bi mice lines CD69Bi.1.7, CD69Bi.2.3, CD69Bi.2.7, CD69Bi.2.8. Flow cytometry analyses of different tissues of 6-8 week old TG mice compared with non-TG littermate. A) Histograms of CD69 (*left*) and GFP (*right*) of non-stimulated (*top*) and stimulated (*bottom*) splenocytes. B) Histograms of CD69 (*left*) and GFP (*right*) of thymocytes. See legend for colors.

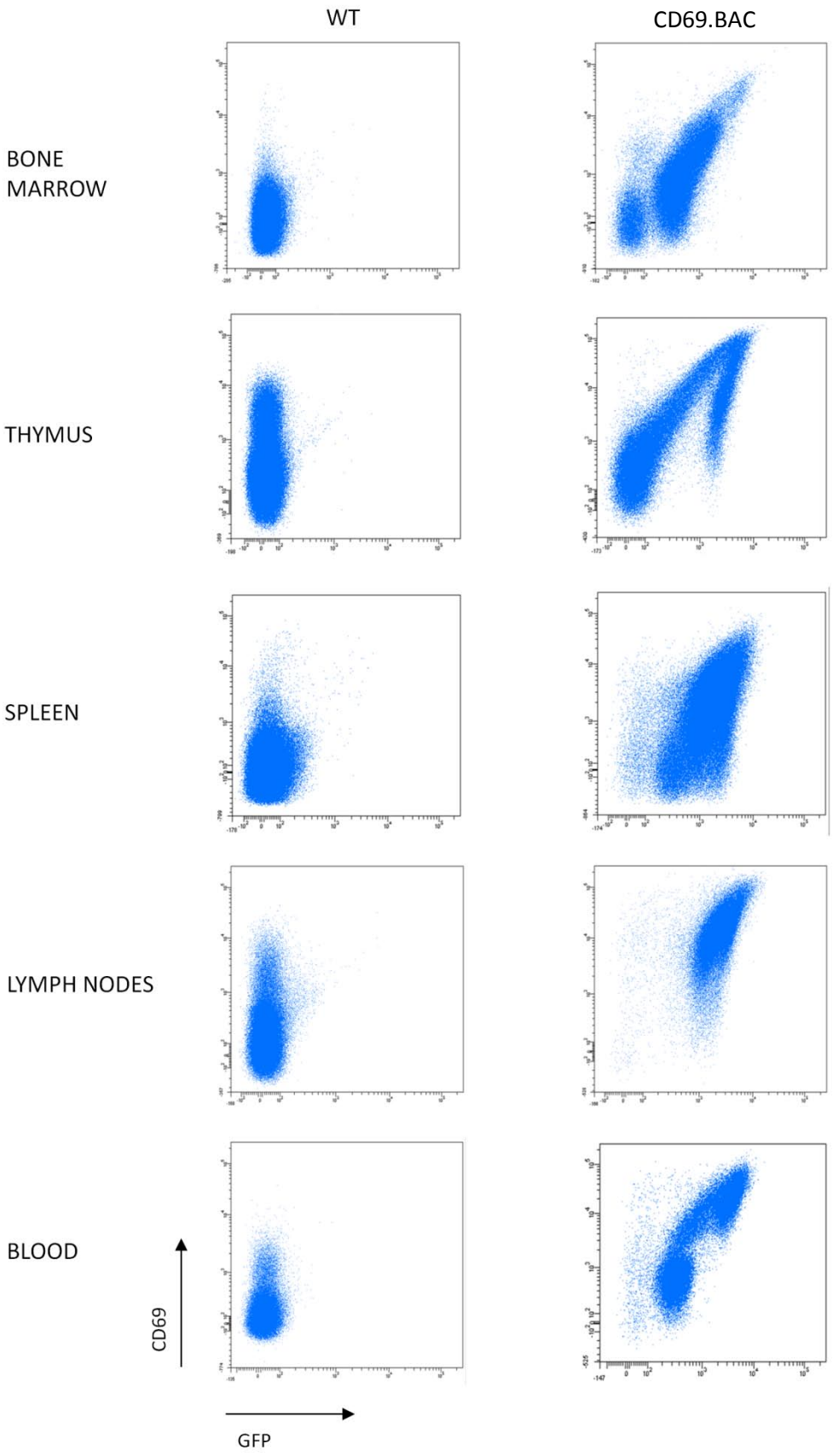


Fig. II.R.3. CD69 and GFP expression in lymphoid tissues of *CD69.BAC* transgenic mice. CD69 vs. GFP plots obtained with flow cytometry of bone marrow, thymus, spleen, lymph nodes and blood of *CD69.BAC* TG mice and wild-type (WT) littermates are shown. Representative plot of eight individuals analyzed of each line.

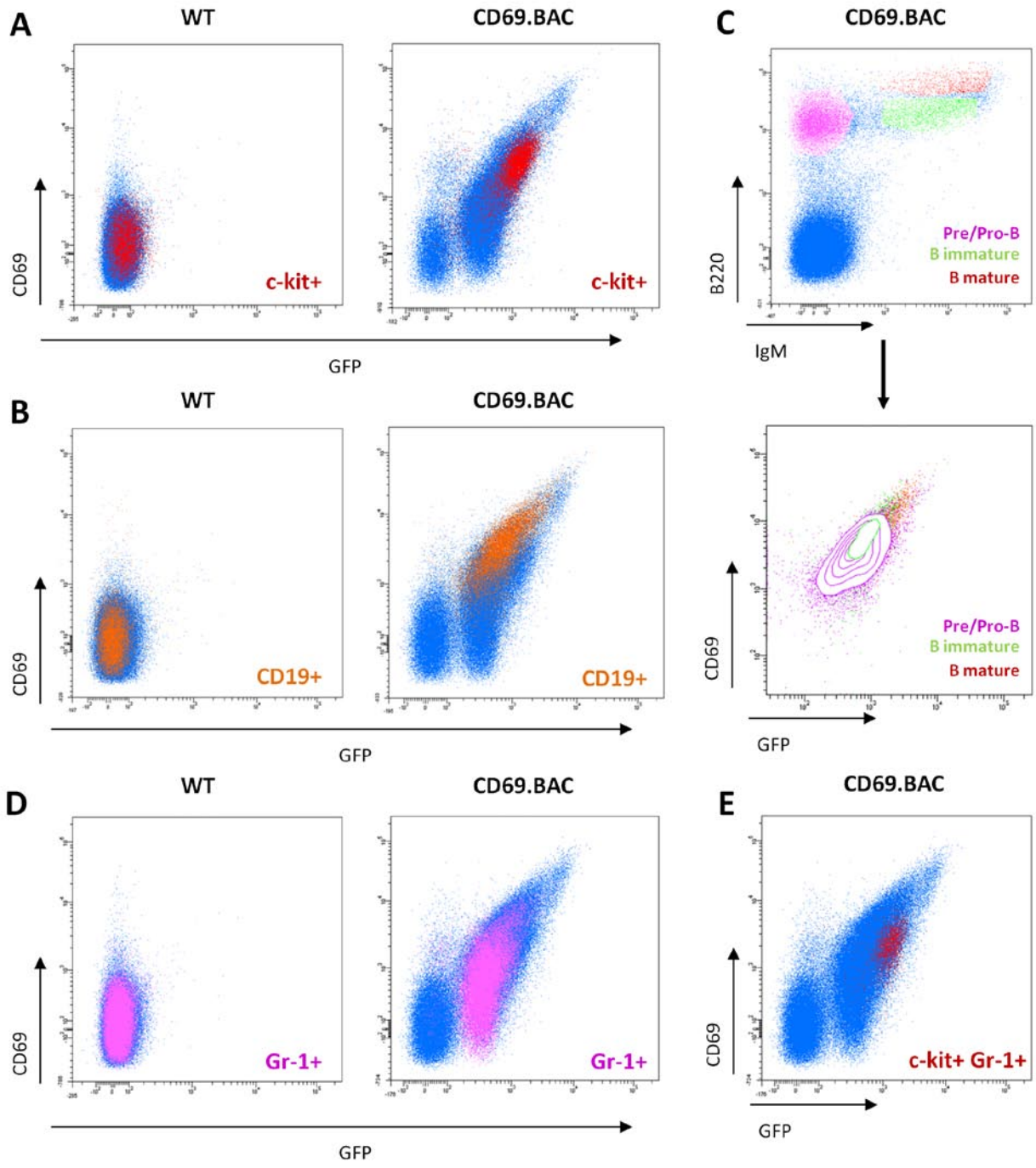


Fig. II.R.4. CD69 and GFP expression in *CD69.BAC* transgenic BM. Distribution of hematopoietic precursors (c-kit+) (A), B cells (CD19+) (B), Granulocytes (Gr-1+) (D) populations of bone marrow of TG mice in CD69 vs. GFP plots obtained with flow cytometry. A, B & D) *Right,* plots of *CD69.BAC* mice; *left*, plots of wild-type littermate mice. C) *Top*, Pre/pro-B cells (pink), immature B cells (green), mature B cells (red) are gated according to their B220 and IgM expression and are shown in CD69 vs. GFP plot in *bottom*. E) CD69 vs. GFP plot of immature granulocytes of *CD69.BAC* mice (Gr-1+ c-kit+). Data are representative of individuals of a total of 8 distributed in 3 experiments.

In contrast, T cells present a different pattern of transgene expression. While they develop in the thymus, CD69 transgenic expression seems to be preceded by endogenous CD69 alleles, as GFP becomes expressed only in CD69 low expressing cells (Fig. II.R.6A).

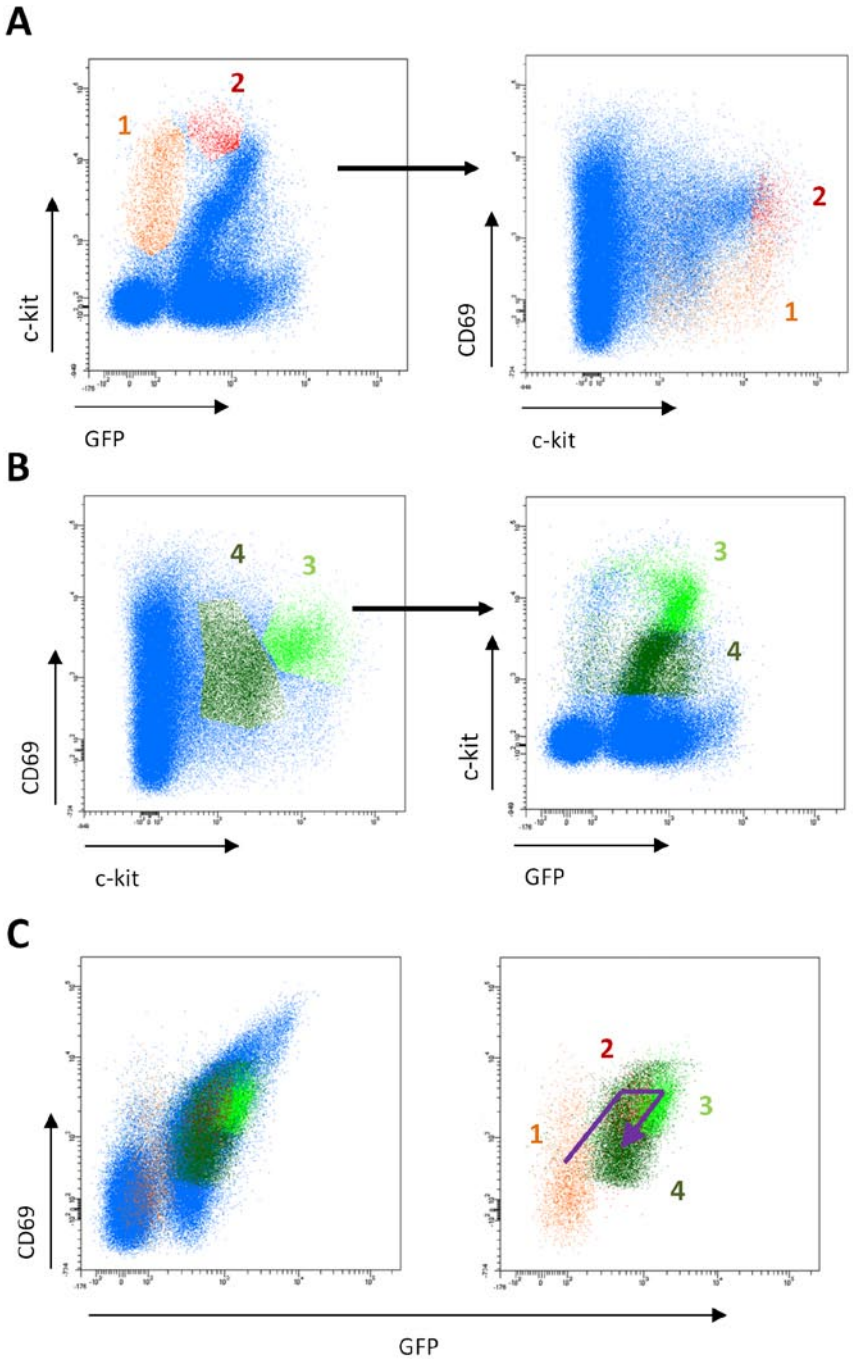


Fig. II.R.5. Sequence of CD69 and GFP expression of hematopoietic precursors in *CD69.BAC* transgenic BM. A, B) *Left,* gated subpopulations in A) c-kit vs GFP plots (1 & 2, in orange and red, respectively) and B) CD69 vs c-kit plots (3 & 4, in light green and dark green, respectively) are displayed in *right*, and then plotted in C). The proposed sequence is marked with a purple arrow

CD4- CD8- cells are CD69- GFP- except as B or dendritic cells (Fig. II.R.6B). An expression sequence could be hypothesized: 1) expression of endogenous CD69 at double positive (DP) stage, 2) expression of the transgene later at this stage, 3) increasing of transgene expression, both CD69 and GFP, 4) slight down-regulation of CD69 but remaining high expression of GFP in the SP stage. In lymph nodes, spleen, bone marrow and blood, T cells show high levels of both proteins, having slightly more CD69 protein the CD4+ than the CD8+ T cells (Fig. II.R.6C).

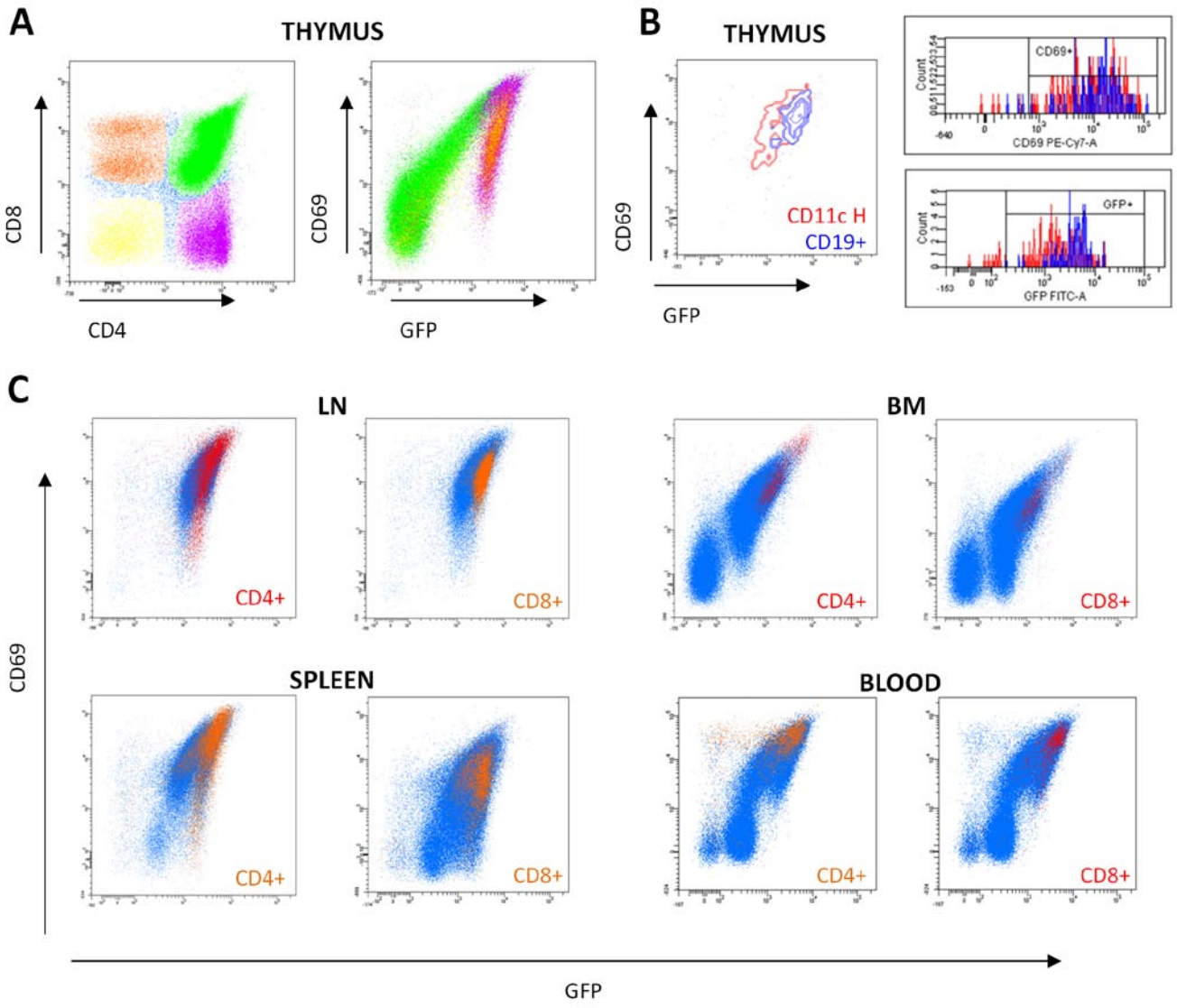


Fig. II.R.6. CD69 and GFP expression on CD4+ and CD8+ T cell populations in *CD69.BAC* transgenic mice. A) *Left*, CD8 vs. CD4 plot with colored subpopulations in thymus of TG mice: CD8SP (orange), CD4SP (pink), double-positive (green) and double-negative thymocytes (yellow). The CD69 vs. GFP distribution of those subpopulations are shown in the *right* panel. B) The CD69 vs. GFP distribution of B cells (CD19+, blue) and dendritic cells (CD11c high, red) in the thymus are marked in the left panel. *Right*, histograms of CD69 (*top*) and GFP (*bottom*) expression of both subpopulations depicted with the same colors as in *left*. C) CD69 vs. GFP plots of CD4+ T (*left*) and CD8+ T cells (*right*) (depicted in orange or red) of different sites: LN (lymph nodes), spleen, BM (bone marrow) and blood. Data is representative of individuals of a total of 8 distributed in 3 experiments.

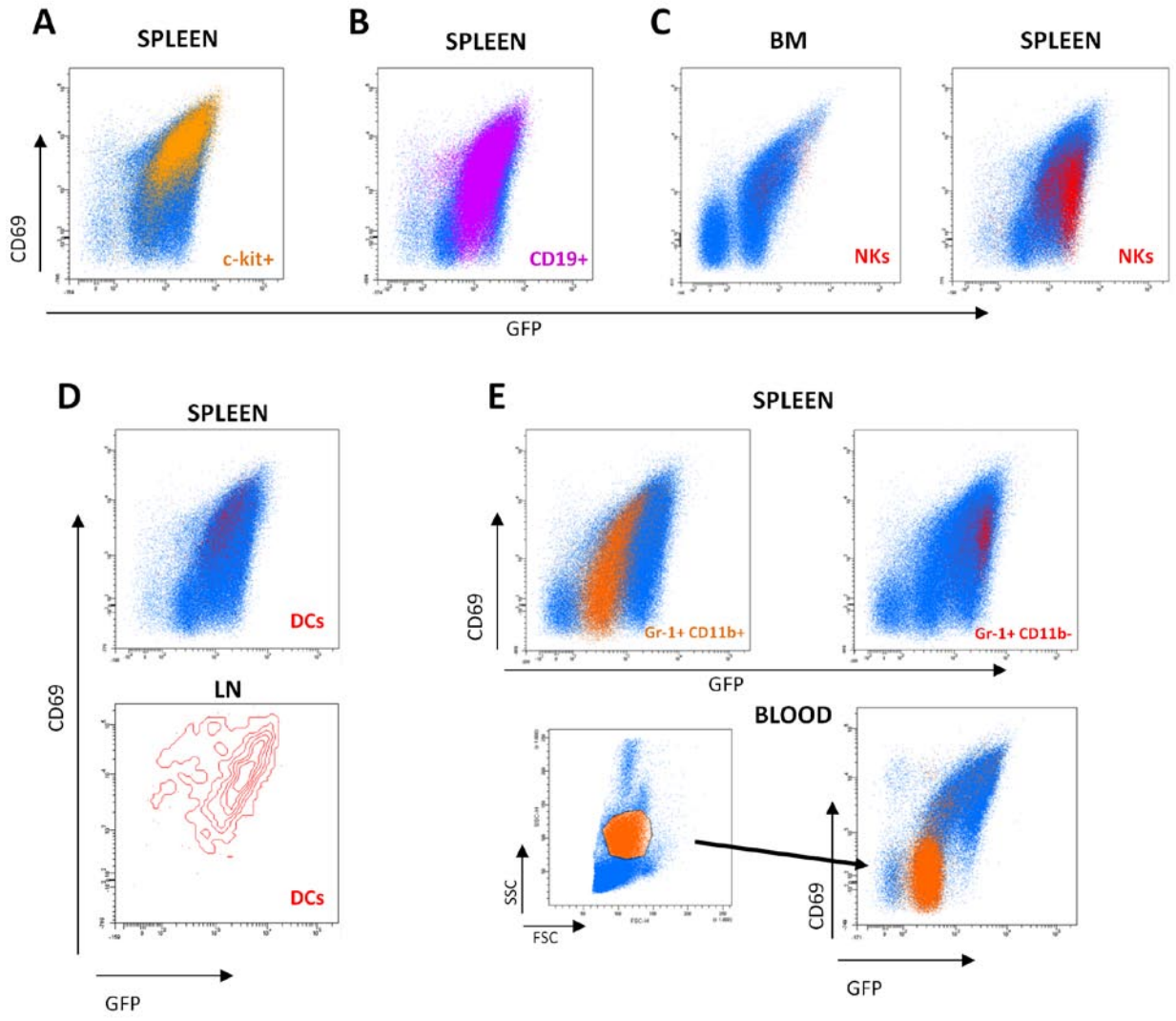


Fig. II.R.7. CD69 and GFP expression of myeloid and B cell populations in *CD69.BAC* transgenic mice. c-kit+ (*A*) (in orange) and CD19+ (B cells) (*B*) (in pink) splenocytes are shown. *C)* NK cells (red) of TG bone marrow (*left*) and spleen (*right*) are depicted. *D)* Dendritic cells of spleen (*top*) and lymph nodes (*bottom*) are shown in red. *E)* CD69 vs. GFP plots for granulocytes. *Top*, spleen granulocytes: *left*, Gr-1+ CD11b+ cells (orange); *right*, Gr-1+ CD11b- population (red). *Bottom*, blood granulocytes defined for their size/complexity properties (FSC/SSC plot, *left*) are depicted in orange in CD69 vs. GFP plot (*right*). Data is representative of individuals of a total of 8 distributed in 3 experiments.

C-kit+ precursors maintain their intermediate transgene expression in spleen (Fig. II.R.7A). Mature B cells (CD19+), as T cells, seem to begin expressing endogenous CD69, then to increase their transgene expression, and finally, to down-regulate CD69 and keep GFP at intermediate levels (Fig. II.R.7B).

Expression of CD69 and GFP in NK cells (DX5 high) is intermediate in bone marrow and becomes higher for the fluorescent protein in spleen, but CD69 expression remains similar (Fig. II.R.7c). DCs show intermediate levels of transgene expression in the thymus (Fig. II.R.6B), LNs and spleen (Fig. II.R.7d).

Granulocytes in spleen and blood are able to down-regulate CD69 and stay with low levels of GFP, as CD69 expression is intermediate in c-kit^{low} Gr-1+ in BM (not shown) and is reduced to almost no CD69 expression in c-kit-Gr-1+ in spleen and blood (Fig. II.R.7E).

There is a small population (< 5%) which shows intermediate/high expression of CD69 but no expression of GFP, as it could be appreciated in Figure II.R.8. It is mainly composed by lymphocytes (B in spleen and LNs and T in the blood). Considering its properties, like a small size in FSC/SSC plot and its dim staining of 7AAD (data not shown), there is the possibility that CD69 expression plays a role in apoptosis, as apoptotic cells can be measured by 7AAD^{dim} staining, while necrotic cells display a 7AAD^{bright} staining (285).

In summary, each population of leukocytes studied shows a different pattern of CD69 and GFP expression, indicating that *CD69.BAC* mice, apart from not being capable of maintaining endogenous CD69 levels, shows singular regulation patterns that may be produced by different types of regulatory mechanisms.

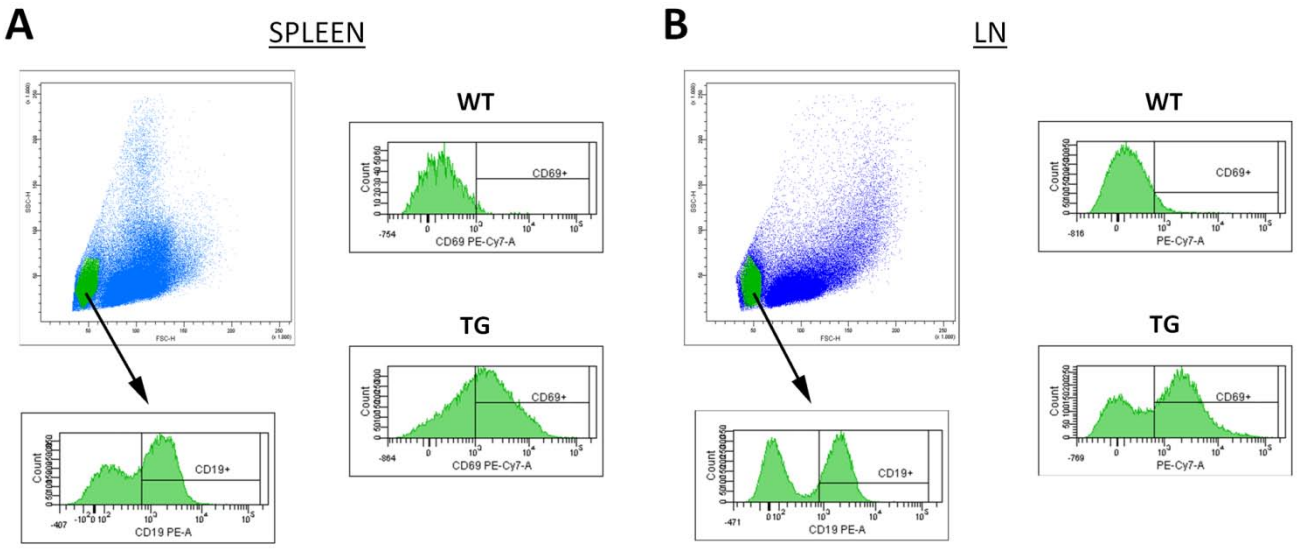


Fig. II.R.8. Low size/complexity cells express CD69 in *CD69.BAC* transgenic mice. Populations with low size/complexity properties (green populations in FSC/SSC plots - *top left* -) in spleen (A) and lymph nodes (B) express mainly CD19 marker in TG mice (*left*). In TG mice these populations also express CD69 (*right*). Percentages of positive CD19 or CD69 cells are depicted on histograms. Data is representative of individuals of a total of 8 distributed in 3 experiments.

Generation of Jurkat-CD69-NLAP and Jurkat-CD69-CLAP

In order to discard positional effects of insertion of BAC transgenes that may have occurred in BAC-in TG mice, we established BAC-CD69-GFP TG cell lines, where multiple clones might be obtained from each transfection with the aim that clones with a transgene expression paralleling endogenous CD69 could be selected.

To optimize the BAC transfection protocol, that is, to establish optimum concentrations of BAC and transfection reagent, a first attempt to set-up an experiment to transfect 2 positive-control BACs was performed. Both BACs are based in the C-LAP cassette (Fig. II.M.4, Section II.M), which contain the GFP protein fused to their C-terminal end of the gene of interest and *neo* gene for selection in mammal cell cultures. One of these BACs (3276) contained the mouse gene *Srsf4,* (serine/arginine-rich splicing factor 4), which is an mRNA splicing factor. *H2afz*, the gene sequence situated in the other BAC (named 3715), codifies for the histone 2A member Z. After trying different transfection conditions, positive clones selected with G418 expressed different amounts of GFP (Fig. II.R.9). Whereas transfection of BAC-3276 gave rise to few GFP(low)+ cells (4%); JJ-3715 line expressed GFP in higher amounts and with two different intensities, GFP^{high} (5%) and GFP^{low} (9%), forming a total of 14% (Fig. II.R.9).

For CD69-expression analyses, two constructs containing fusion proteins with GFP for detection and *neo* gene for selection of mammal cell culture were generated: CD69-NLAP, which contained the appropriate sequences to obtain a BAC with GFP fused to mouse CD69 in its N-terminal end; and CD69-CLAP, where the final BAC would be composed of a CD69-GFP construct fused by the C-terminal end (Fig. II.M.4, section II.M). Each construct was recombined to 3 different BACs, obtaining 6 BACs: N171I2 (N-terminal fusion of GFP) & C171I2 (C-terminal fusion of GFP) from BAC RP24-171I2; N188C4 & C188C4 from BAC RP24-188C4; N109H21 & C109H21 from BAC RP24-109H21.

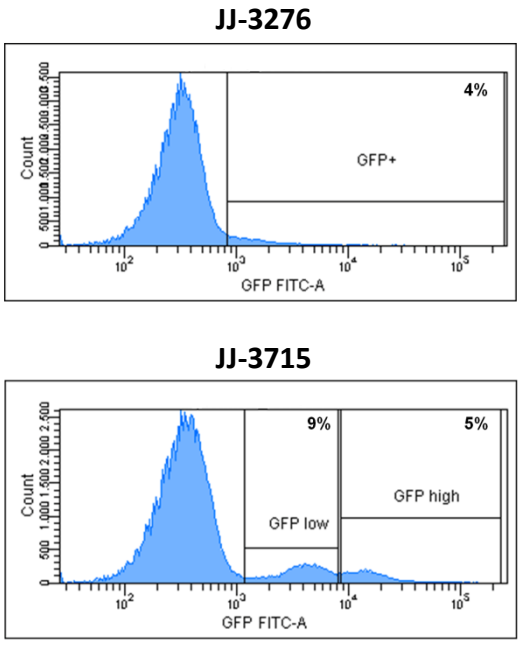


Fig. II.R.9. Expression of GFP of Jurkat cell clones transfected with positive-control BACs. GFP histograms obtained with flow cytometry of bulk Jurkat cell transfected with BAC-3276 construct (JJ-3276) and BAC-3715 (JJ-3715). Percentages of GFP+, GFP-low and GFP-high cells are shown.

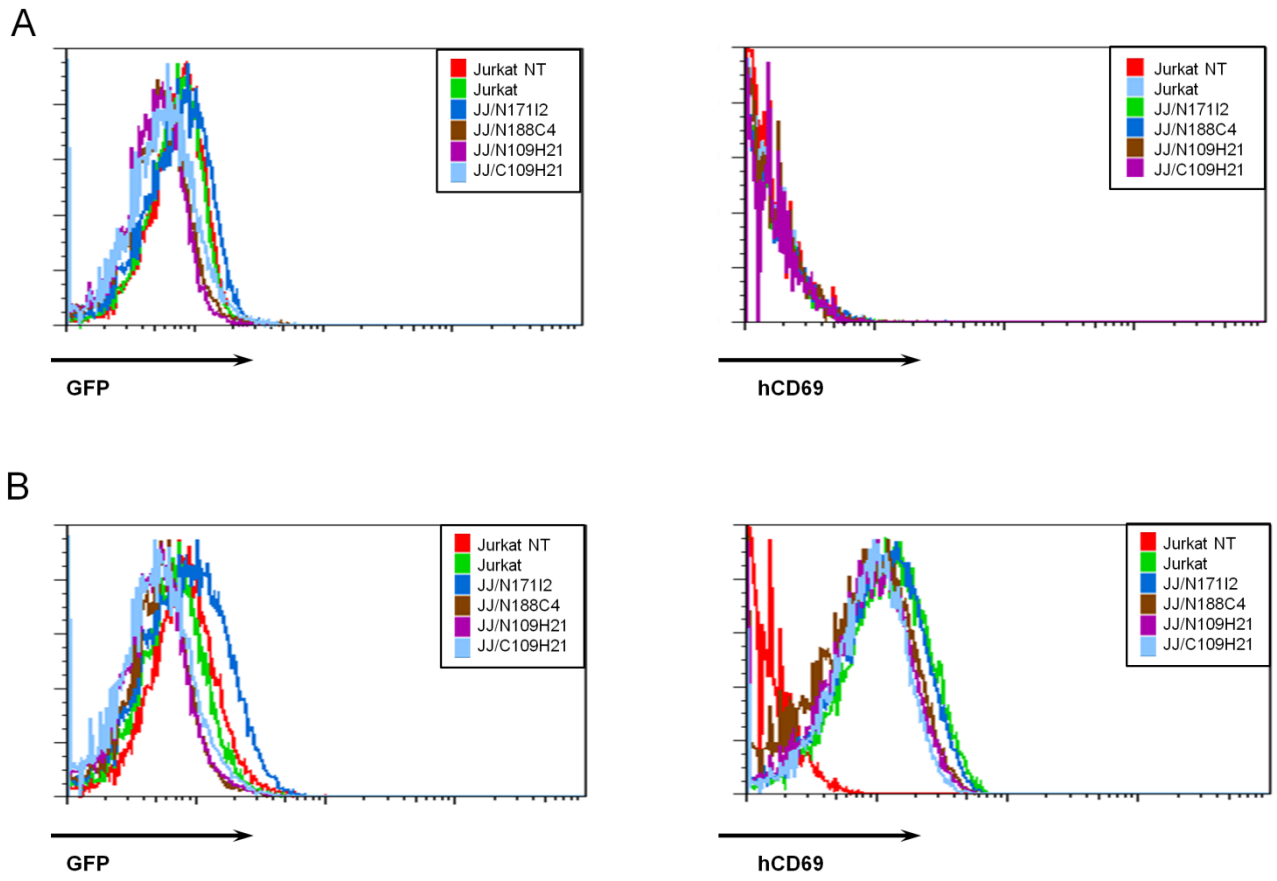


Fig. II.R.10. GFP and human CD69 expression of Jurkat cells transfected with BAC-CD69-GFP constructs. Jurkat cells were transfected with modified BACs containing GFP cDNA fused to CD69 and selected for *neo* resistance during 15 days. Surviving clones were isolated and tested for GFP and hCD69 expression by flow cytometry. Histograms displayed for A) non-stimulated or B) 24h PMA-stimulated cells. *Jurkat NT*, non-stained non-transfected Jurkat cells; *Jurkat*¸ stained non-transfected Jurkat cells; *JJ/N171I2*, Jurkat transfected with BAC RP24-171I2 with GFP inserted in N-term of CD69 gene; *JJ/N188C4*, Jurkat transfected with BAC RP24-188C4 with GFP inserted in N-term; *JJ/N109H21*, Jurkat transfected with BAC RP24-109H21 with GFP inserted in N-term; *JJ/c109H21*, Jurkat transfected with BAC RP24-109H21 with GFP inserted in C-term. Data are representative of two experiments.

Recombined and purified BACs were then transfected into Jurkat cell line applying identical conditions as used formerly for BAC positive-controls. Several attempts were performed employing different transfection reagents and none of the G418-selected clones expressed GFP in basal state or under stimulation (Fig. II.R.10). In contrast, transfection in CHO cells did give rise to GFP expressing cells at high levels and in all clones transfected with any of the BACs employed (*JJ/N171I2, JJ/N188C4, JJ/N109H21* and *JJ/c109H21)* (Fig. II.R.11). These results indicate that expression restrictions which were encountered *in vivo* and in Jurkat cells are overridden in this cell line. An outstanding observation is that GFP^{mid} and GFP^{high} cells do not express CD69 on the cell surface, suggesting that some impediment blocks the CD69 expression of the

transgene on membrane. Only, in a small population of CD69/GFP transfected CHO line, CD69 is detected and is coincidental with GFP^{dull} detection.

Furthermore, there is a small population (<1%) that express mCD69 cells also in non-transfected CHO cells (Fig. II.R.11, top left), indicating anti-mCD69 reacts with the endogenous CD69 of CHO cells, that are derived from hamster.

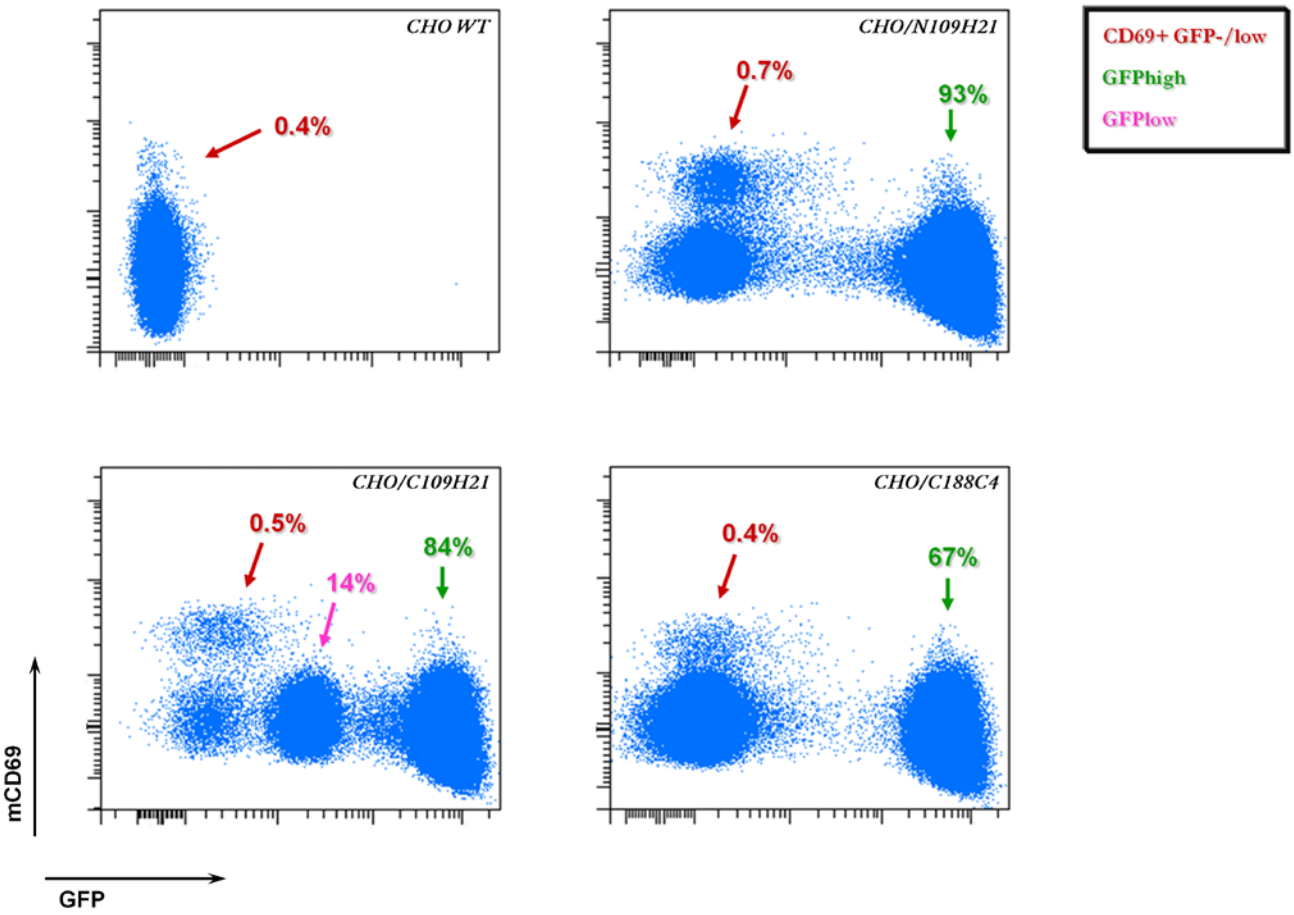


Fig. II.R.11. GFP and mouse CD69 expression of CHO cells transfected with BAC-CD69-GFP constructs. CHO cells were transfected with modified BACs containing GFP cDNA fused to CD69 and selected for *neo* resistance during 15 days. Surviving clones were isolated and tested for GFP and mCD69 expression by flow cytometry. mCD69 vs GFP plots are displayed for non-transfected CHO cells (*top left, CHO WT*), CHO transfected with BAC 109H21 with GFP fused in N-term of mCD69 (*top right, CHO/N109H21*), CHO transfected with 109H21 with GFP fused in C-term (*bottom left, CHO/C109H21*), and CHO transfected with BAC RP24-188C4 with GFP fused in C-term (*bottom right, CHO/C188C4*). Percentages of each population are marked in different colors (see Legend). Data are representative of two experiments.

DISCUSSION

In this chapter, we have generated four CD69- BAC-in- TG mice lines and several clones of six CD69-BAC-in TG mammal cell lines (including Jurkat and CHO cells, using the method of Poser et al. commented previously), obtaining no regulated models of TG CD69 expression. One of four lines of TG mice expressed the transgene in higher levels that endogenous CD69, although with specific expression in each type of leukocyte. In TG cell lines, a high expression of GFP (but no mCD69) was observed in BAC-transfected CHO cells, in contrast to no expression noted in Jurkat cells.

BAC transgenesis has been proved to be a reliable method to study protein regulation and function (251-258). However, the transgenic expression of some proteins was not achieved using BAC transgenesis in cell lines first established by Poser et al. (262). They observed a 60% of transgenes expressed from the total genes assayed, rising to an 85% only considering the successfully transfected BAC genes. Nevertheless, they did not specify reasons for this 15% of non-expressing clones, which may be explained by different elements and/or mechanisms that BACs do not provide and they do not confirm the proper regulation of the transgenes.

The lack of proper regulation of all transfected mice/cell lines may be explained by abnormal transcriptional regulation occurring in the transgene locus, despite it containing the putative majority of the *cis*-acting elements. This transcriptional regulation may be controlled by chromatin accessibility, which was previously suggested to be specifically high in CD69 regulatory regions in hematopoietic cells (Chapter I). However, these data do no correlate with the results we obtained in BAC transgenesis in cell lines, as the non-hematopoietic CHO cell lines expressed the transgene in all the G418-selected clones and no expression was observed in any of the clones of Jurkat cell line, suggesting that other type of regulatory mechanisms are participating. In fact, the consistency of the results of the transgene expression in all the positive-selected clones of CHO transfected cell lines, mostly discard transgene deregulation caused by positional effects, although it must be considered that chromatin properties might be different in distinct cell types, as CHO cell line is widely employed in recombinant protein production (286).

In contrast, a specific developmental regulation of CD69 expression seems to occur in thymocytes of *CD69.BAC* TG mice. Data from Figure II.R.6.A points that DP thymocytes express transgene after

endogenous CD69 gene, suggesting a locus directed expression, that may be produced by the establishment of chromosome territories that can be developmental and lineage specific (287, 288), as occurs in WT mice (289, 290). Therefore the different expression observed in thymocytes of CD69-overexpressing mice and in Jurkat cells, might be caused by the existence of a developmental chromosome territory. As expression of GFP is noted in some of the TG mice and cell lines, we propose there are enough *cis*-acting elements to produce transcription. Whether or not these elements are enough to produce CD69 transcription in every cell type studied remains to be determined. The absence in the transgene of some types of these elements, like Locus Control Regions (LCRs) (which may be located hundreds of kilobases away from the gene -reviewed in (291)-), might explain this abnormal regulation of expression. Nevertheless, the high expression observed in the TG mice with high copy number of the transgene and the high expression of GFP in transfected CHO cell line point to additional regulatory mechanisms are occurring, as postranscriptional events, unless that the presence of high copy numbers of the trasngene could override the lack of the LCR.

It has been shown CD69 mRNA is degraded rapidly after cell stimulation and its 3' UTR contributes to destabilize it (146). In our TG mouse lines, this type of destabilization may be occurring except in the *CD69.BAC* TG line, as a high-copy number of the transgene could give rise to large intracitoplasmic RNA amounts difficult to degrade. An additional mRNA degradation mechanism might be occurring caused by the high length of the transgenic mRNA, as mature mRNA rises from 1.6 kb of WT gene to 3 kb of transgenic gene.

An interesting observation in the *CD69.BAC* TG line was to find that myeloid cells lines (Gr-1+) showed a different transgene expression than the one of lymphoid (T, B and NK cells). The former, upon maturation, were able to down-regulate CD69 expression to almost the same as WT littermate mice while transgenic maturing lymphocytes could only slightly downregulate it. A possible explanation, as CD69 expression in myeloid cells was defined to be slower (73, 74, 77, 78), is that the mRNA degradation machinery is capable to function properly in these cells and not quick enough in lymphocytes. In addition, stabilization mechanisms that were previously described in other tight regulated immune proteins, could act in a different manner between lymphoid and myeloid lineages. Also, RNA degradation may be mediated by micro-RNAs (292, 293) In this line, it was recently discovered that CD69 expression can be regulated by different micro-RNAs (147, 148), and data from *TargetScan* (http://www.targetscan.org) reveals 3' UTR region having several binding sites for miRNAs (see Fig. D.1, Section General Discussion).

Post-translational events may also be controlling CD69 expression and may cause the differences observed in CD69 expression in the *CD69.BAC* TG mice. A mechanism of post-translational regulation of surface CD69 mediated by the sphingosine-1-phosphate receptor 1 (S1P1) has been proposed (149, 150), as there are evidences of direct interactions (112, 151). In this model, S1P1 binds CD69 on membrane and both proteins are internalized, resulting in that high S1P1-expressing cells would not express CD69 on membrane. In the same way, an excess of CD69 expression would downregulate S1P1. Although it was firstly proposed for lymphocytes, new evidences implicate CD69 in cDC and hematopoietic precursor trafficking mediated by S1P1 (294, 295). This model can help to explain the different levels of CD69 and GFP in *CD69.BAC* mice leukocytes, in particular in T cells and granulocytes. Moreover, GFP has been reported to be a highly stable protein (296, 297) that cannot be easily down-regulated. Taking these two things into account, it can be hypothesized that in our transgenes, the fact that the observed levels of GFP do not parallel the ones of CD69 is not due to divergent translational regulation but to that CD69 is expressed but not present on the cell membrane whilst the GFP continues to be detectable in the cytoplasm for a long time.

In conclusion, transgenesis of BAC containing CD69 pointed for the need for additional elements or mechanisms, which are absent in the genomic context provided by CD69- BAC, for proper regulation of CD69 expression. These mechanisms seem to include transcriptional, post-transcriptional and post-translational events, although plenty of new studies involving chromosome territory (3C assays), RNA regulation (real-time PCR or Northern Blots) and protein expression (S1P1 levels of RNA/protein, intracitoplasmic staining) are required in order to confirm the relative participation in specific cells and tissues.

CHAPTER III:

ALTERED LEUKOCYTE DISTRIBUTION BY DISREGULATION OF CD69 EXPRESSION

CAPÍTULO III:

DISTRIBUCIÓN DE LEUCOCITOS ALTERADA POR DISREGULACIÓN DE CD69

INTRODUCTION

CD69 is an immune-related receptor known since the mid 80's (67); however, its biological function is not well established. Several clues related it to, on one hand, regulation of immune balance between activation and inhibition of responses (reviewed in (298)), and in the other, control of leukocyte migration (112, 150).

The first studies of CD69 function *in vitro* revealed an important role in leukocyte activation (reviewed in (246, 298)), being proposed as a co-stimulatory molecule, as its triggering elicited different signals of activation. Furthermore, function in controlling the immune response has been proposed in some studies *in vivo*, due to the exacerbated response observed in KO mice in different models (89, 92, 94-96, 98). Moreover, CD69 has been proposed to be related with Th17 and Treg populations, favoring the control of the immune activation (102-106).

Recent studies on CD69 function are focused on its role in leukocyte migration. TG mouse lines where CD69 is overexpressed in T cells (CD69 cDNA transgene directed by *Lck* promoter, (110) and by hCD2 promoter and enhancer, (111)) showed increased proportions of single-positive thymocytes in thymus. In both studies, these SP populations showed a mature phenotype; although also isolated effects on thymocyte selection (110) and proliferation in response to *in vitro* stimuli (111) were observed. The effect on thymocyte development was proposed to be caused by enhanced migration across the thymus compartments, from cortex to medulla (110). In addition, CD69 -/- models also revealed altered migration patterns. CD69KO1 mouse line showed increased cellularity in spleen and peritoneum (96). CD69KO2 mice showed enhanced migration of thymocytes from thymus to lymph (120) and recently, exhibited deficient relocation and persistence of memory T helper (Th) population in bone marrow (121).

These data are in agreement studies establishing a relation between CD69 and S1P1 (sphingosine-1-phosphate receptor 1). S1P1 is a G-coupled receptor responsible for lymphocyte migration (114, 115). This receptor is upregulated to allow the exit of lymphocytes from the thymus and the lymph nodes to migrate to the blood and the lymph, respectively, where the concentration of its ligand, S1P, is more elevated, producing a chemotactic effect. It has been proved that expression of CD69 inhibits surface S1P1 expression on lymphocytes and causes lymphocyte retention in lymphoid organs (112). CD69 is also inhibited by S1P1 triggering (299-302), acting through the transcription factor HIF-1α (102).

Regarding B-cell migration, deficient trafficking due to the lack of S1P responsiveness was detected in CD69KO2 mice (112). Furthermore, S1P1 has been considered to participate in immature B-cell population egress from BM to blood (303, 304) and also to be necessary to the homing of plasma cells to BM and egress from LNs (305).

Studies in regulatory T cells (Treg) in lymphocyte-restricted S1P1 -/- and S1P1 tg mice, revealed Treg altered migration, development and differentiation of this subpopulation (306).

As for lymphocytes, an interaction between CD69 and migration of cDCs through S1P1 from skin to lymph nodes under stimuli has been established (294), favoring the retention in skin.

We study in this chapter the distribution of leukocytes in a CD69 overexpressing mouse line, observing important alterations, some of them not previously assessed.

MATERIAL & METHODS

Mice and cells

6 - 12 weeks old mice from *CD69.BAC* mouse lines and WT littermates were sacrificed and several tissues and fluids were harvested: spleen, thymus, blood, brachial, axillary, inguinal popliteal and maxillary lymph nodes (LN) and bone marrow (BM). Spleens of 6 - 8 weeks old C57BL/6 and BALB/c (WT and CD69.KO.1) mice were employed for analysis of Tregs in KO mice. Cells from tissues were released by mechanical disaggregation, washed twice with 1x PBS and pelleted. Blood samples (diluted upon extraction at least 10 times in 1x PBS to avoid coagulation) were centrifuged and the supernatant was removed. After that, all samples were lysed, except for LN cells, with Lysis Buffer (0.15 M NH4Cl, 1mM KHCO3, 1mM Na2-EDTA, pH 7.4) for a maximum of 2 min. Samples were washed twice with 1x PBS and pelleted for immunostaining.

Flow cytometry analyses

Mouse cells were immunostained for analysis by Flow Cytometry. They were previously added purified rat anti-mouse CD16/CD32 (*Fc Block* from Becton-Dickinson) to avoid unspecific binding of antibodies to Fc receptors. Staining was left for 20 min at 4º C with FITC-, PE- APC-, PeCy5- and/or PECy7- conjugated antibodies or biotinilated antibodies, all diluted in staining buffer (complete list of antibodies shown in Table II.M.III -Chapter II-). In samples stained with biotinilated antibodies a second staining was performed with streptavidin- fluorochrome conjugates (Table II.M.III). When necessary, intracellular staining of FoxP3 was performed, after surface staining, employing *Foxp3 / Transcription Factor Staining Buffer Set* from eBioscience Inc., and Anti-Mouse/Rat Foxp3-PE antibody according to manufacturer's instructions. After primary and secondary stainings, cells were washed twice with staining buffer. 7-AAD (BD Pharmingen) was added at the end to exclude dead cells, except for intracellularly stained cells.

Samples were analyzed on *FACSCanto* and *FACSCalibur* flow cytometers (Becton Dickinson) and the software employed to analyze the data was *FACSDiva* (Becton Dickinson) and *FlowJo* (TreeStar Inc.).

Statistical analysis

A two-tailed Student's *t*-test was used for statistical analysis. Differences with a *P* value of less than 0.05 were considered significant.

RESULTS

1. ALTERED LEUKOCYTE DISTRIBUTION IN TRANSGENIC MICE WITH OVEREXPRESSION OF CD69

Reduced cellularity in spleen and lymph nodes of CD69.BAC mice

To study the impact of CD69 overexpression in immune system, we analyzed leukocyte subpopulations in the lymphoid organs and blood of the previously generated *CD69.BAC* mice by flow cytometry. We extracted thymus, spleen, bone marrow, blood and lymph nodes (brachial, axillary, maxillary, inguinal and popliteal) from TG mice and WT littermates. Spleen and thymus were weighted and no significant differences were found between organs from TG and WT mice (Fig. III.R.1.1).

Cells of each tissue were then disaggregated and counted, except for blood. Remarkably, *CD69.BAC* mice showed significantly lower splenocyte and LN cell counts, while no differences were found in cell numbers of bone marrow (BM) and thymus (Fig. III.R. 1.2).

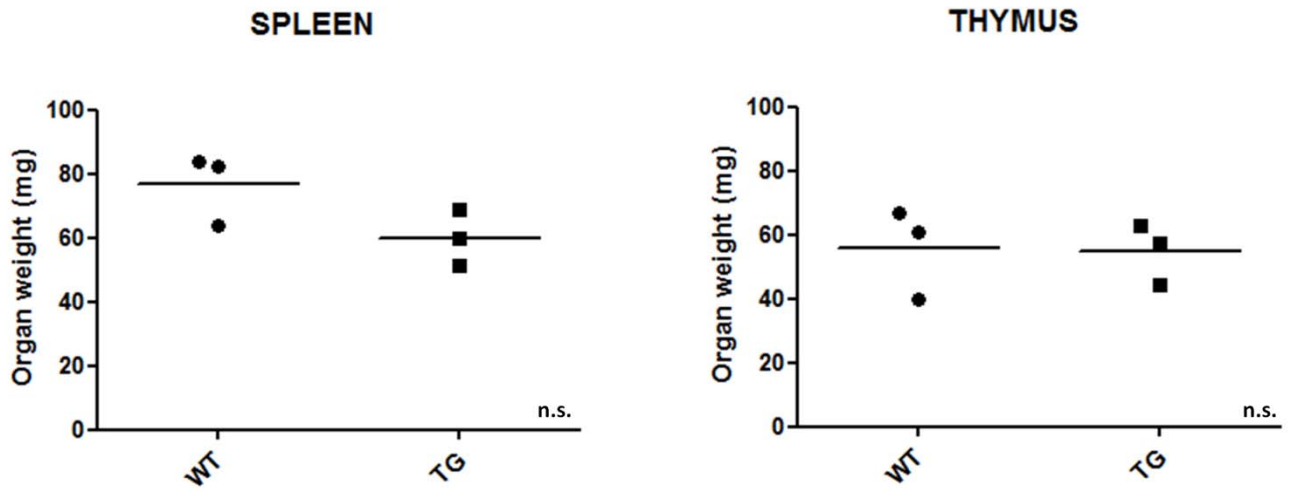


Fig. III.R.1.1. Organ weight of *CD69.BAC* transgenic and wild-type littermates. Weight of spleen (*left*) and thymus (*right* for wild-type (WT) and *CD69.BAC* (TG) mouse lines. (n.s.): non-significant differences) is expressed in milligrams. Data are representative of three experiments.

Mature thymocytes accumulate in the thymus of CD69.BAC mice

Different studies have formerly reported that overexpression of CD69 *in vivo* elicits a mature thymocyte retention in thymus (110, 111). Therefore we examined thymocytes for double-negative (DN), double-positive (DP), and single-positive (CD4SP/ CD8SP) subpopulations. As expected, single-positive CD4 and CD8 thymocytes were significantly augmented in percentage and number (Fig. III.R.1.3A & B, respectively).

To study the possible retention of SP thymocytes, we analyzed T cell percentages and numbers in the periphery. We found considerably reduced (about to the half) T cell numbers in spleen and lymph nodes and diminished in percentage in bone marrow (BM) and blood (Fig. III.R.1.4) of the TG mice when compared to the WT controls, reinforcing the hypothesis of mature thymocyte retention in thymus.

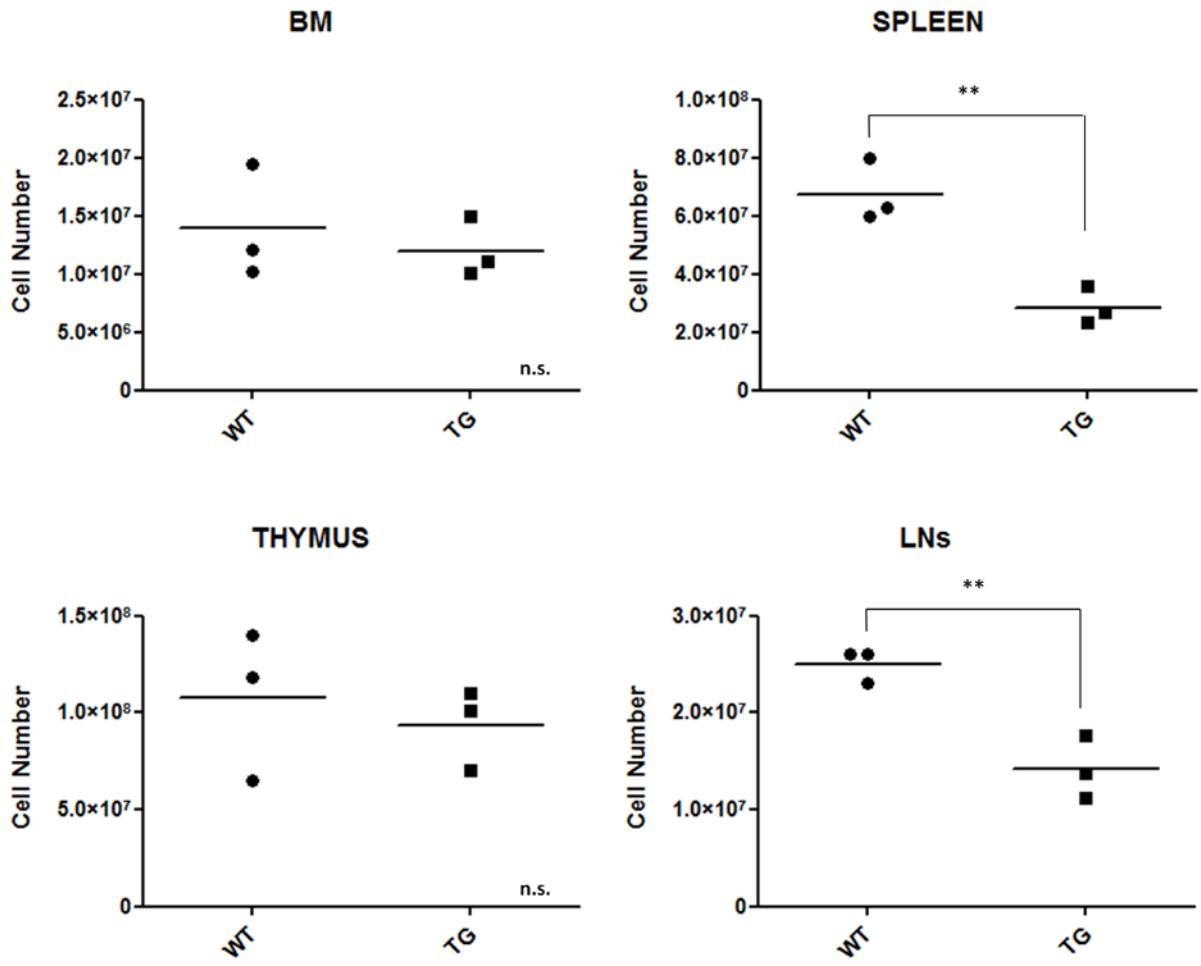


Fig. III.R.1.2. Total cell counts for lymphoid tissues in *CD69.BAC* transgenic and wild-type littermates. T cells numbers for wild-type (WT) and *CD69.BAC* (TG) mouse lines. (BM): bone marrow; (LNs): lymph nodes. (**) $p<0.01$; (n.s.): non-significant differences. Data are representative of two experiments.

B cell dsitribution is altered in CD69.BAC mice

A significant reduction in B-cell numbers was observed in the spleen of tg mice, a slight non significant reduction of these cells also appeared in thymus and bone marrow, and significant lower percentage was seen in blood (Fig. III.R.1.5). In contrast, in lymph nodes (LN) their numbers are similar to wt mice.

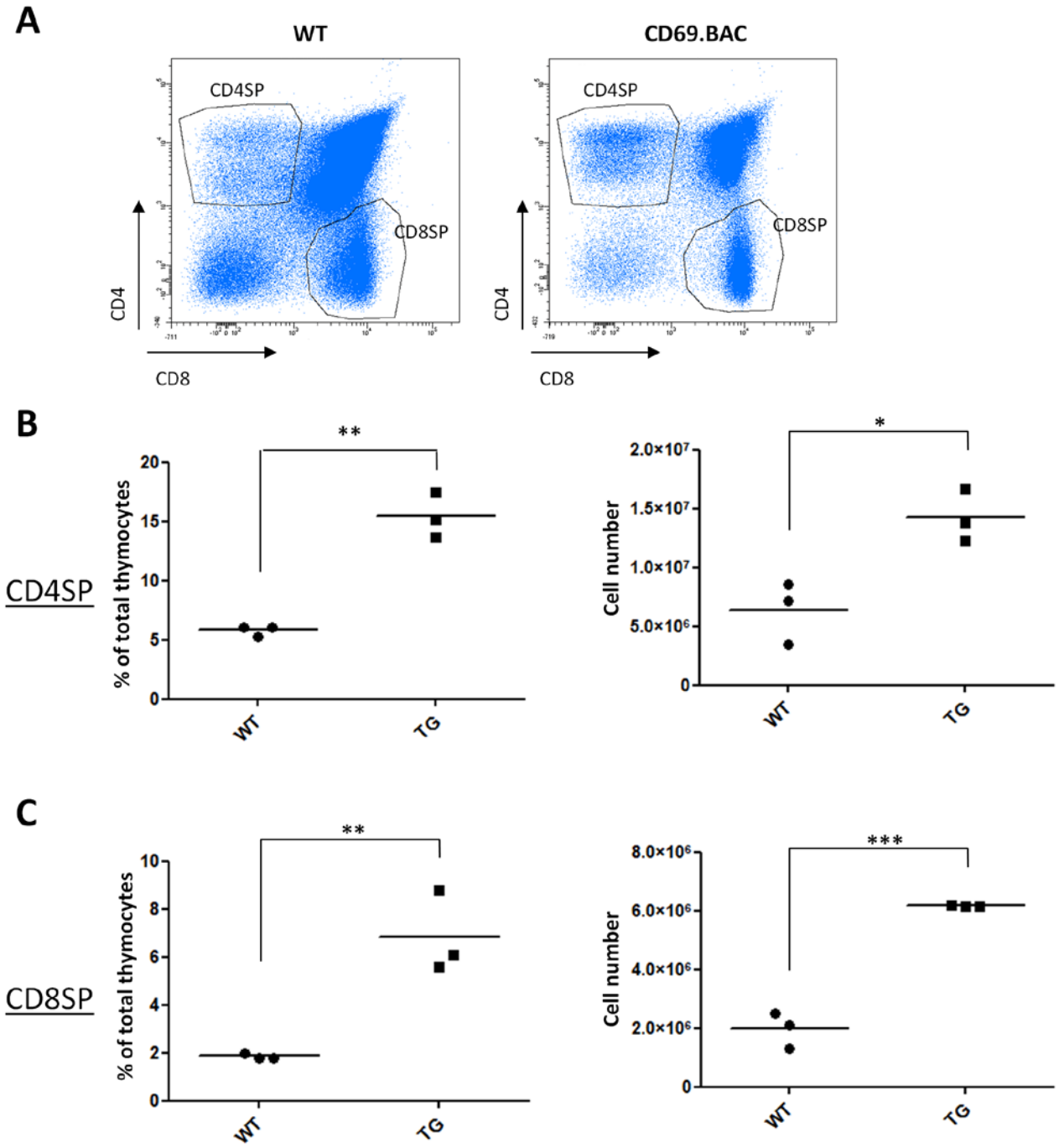


Fig. III.R.1.3. Augmented single-positive thymoctes in *CD69.BAC* mice. A) CD4SP & CD8SP populations from thymus were gated as indicated in wild-type (WT) and *CD69.BAC* mice (CD69.BAC) to analyze percentages and numbers showed in *B* and *C*. B, C) Percentages (*left*) or total numbers (*right*) of CD4SP (B) and CD8SP (C) thymocytes are shown for tg and WT mice. (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$. Data representative of four experiments.

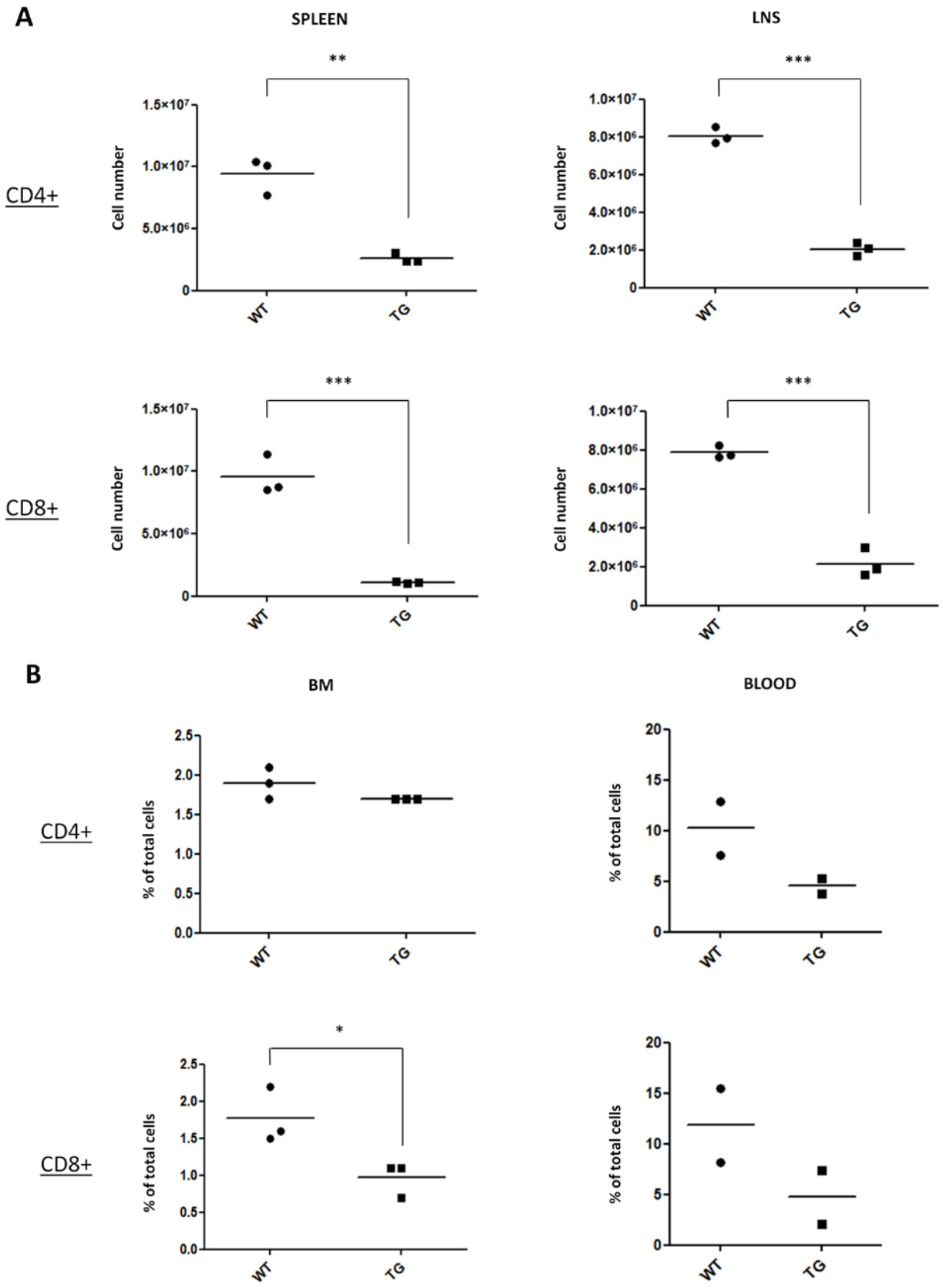


Fig. III.R.1.4. Reduced T lymphocyte presence outside *CD69.BAC* transgenic thymus. (A) Cell numbers of CD4+ (*top*) and CD8+ T cells (*bottom*) in spleen (*left*) or LNs (*right*) wild-type (WT) and *CD69.BAC* mice (TG). (B) Percentages of CD4+ (*top*) and CD8+ T cells (*bottom*) in BM (*left*) or blood (*right*) in WT and tg mice (TG). (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$. Data representative of three to four experiments.

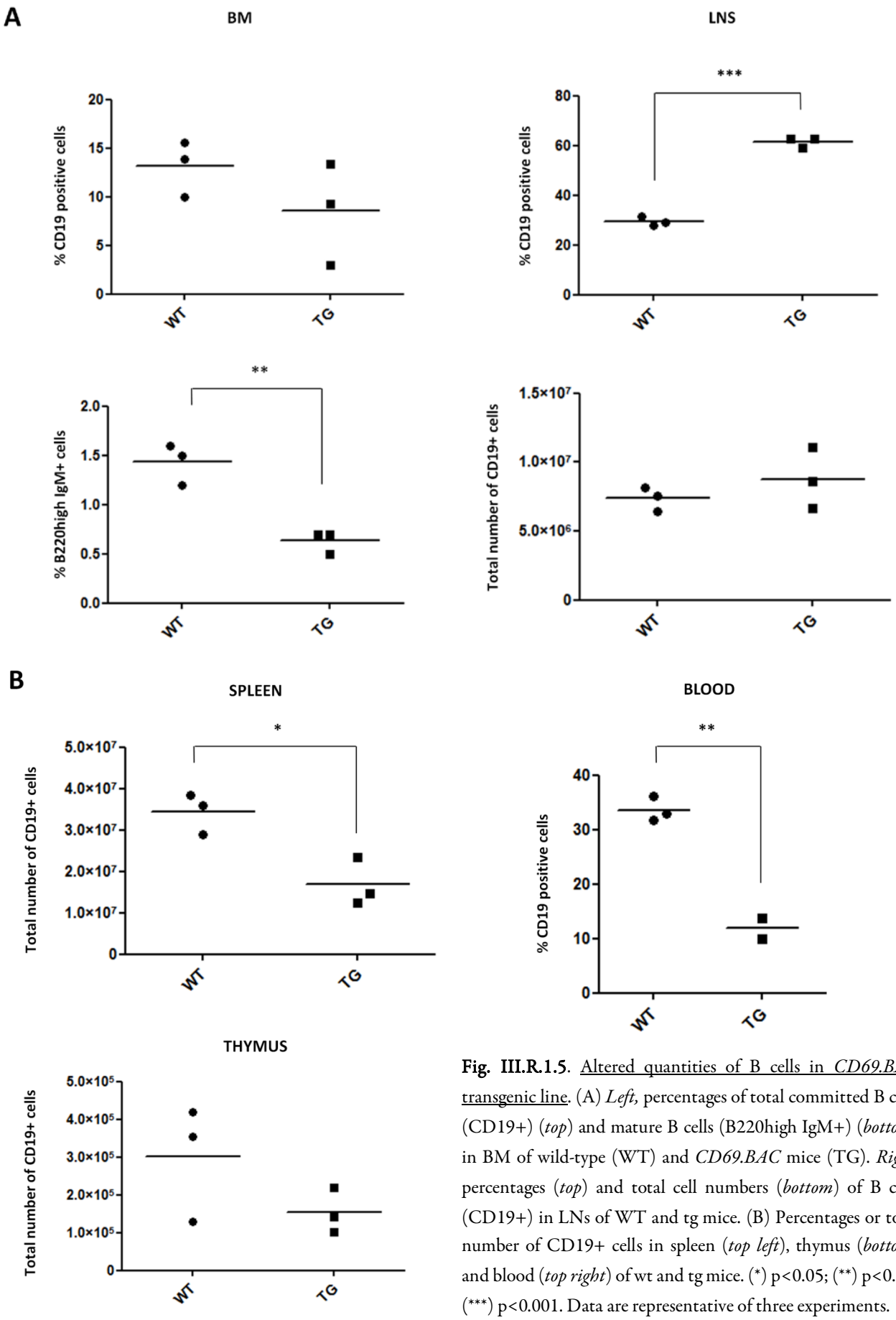


Fig. III.R.1.5. Altered quantities of B cells in *CD69.BAC* transgenic line. (A) *Left*, percentages of total committed B cells (CD19+) (*top*) and mature B cells (B220high IgM+) (*bottom*) in BM of wild-type (WT) and *CD69.BAC* mice (TG). *Right*, percentages (*top*) and total cell numbers (*bottom*) of B cells (CD19+) in LNs of WT and tg mice. (B) Percentages or total number of CD19+ cells in spleen (*top left*), thymus (*bottom*) and blood (*top right*) of wt and tg mice. (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$. Data are representative of three experiments.

An interesting observation was the reduction (significant in percentage) of CD19+ and B220^{high} IgM+ populations but not of immature or pre-/pro- B-cells in BM (Fig. III.R.1.5A) of the *CD69.BAC* mouse. The former population is considered to be a mature subset recirculating in blood and relocating in BM and was found to be reduced in S1P1- deficient mouse lines (115, 303, 304).

Alteration of other subpopulations distribution

Remarkably, a decrease in conventional dendritic cell numbers (defined as expressing high levels of the receptor CD11c (198, 307)), was observed in the tissues studied: spleen, LNs and -non-significantly- thymus (Fig. III.R.1.8).

NK cells (DX5^{high}) show augmented percentages in BM and significantly in spleen of TG mice, but total recount numbers are similar in both tissues (Fig. III.R.1.6), indicating no appreciable alterations in NK distribution.

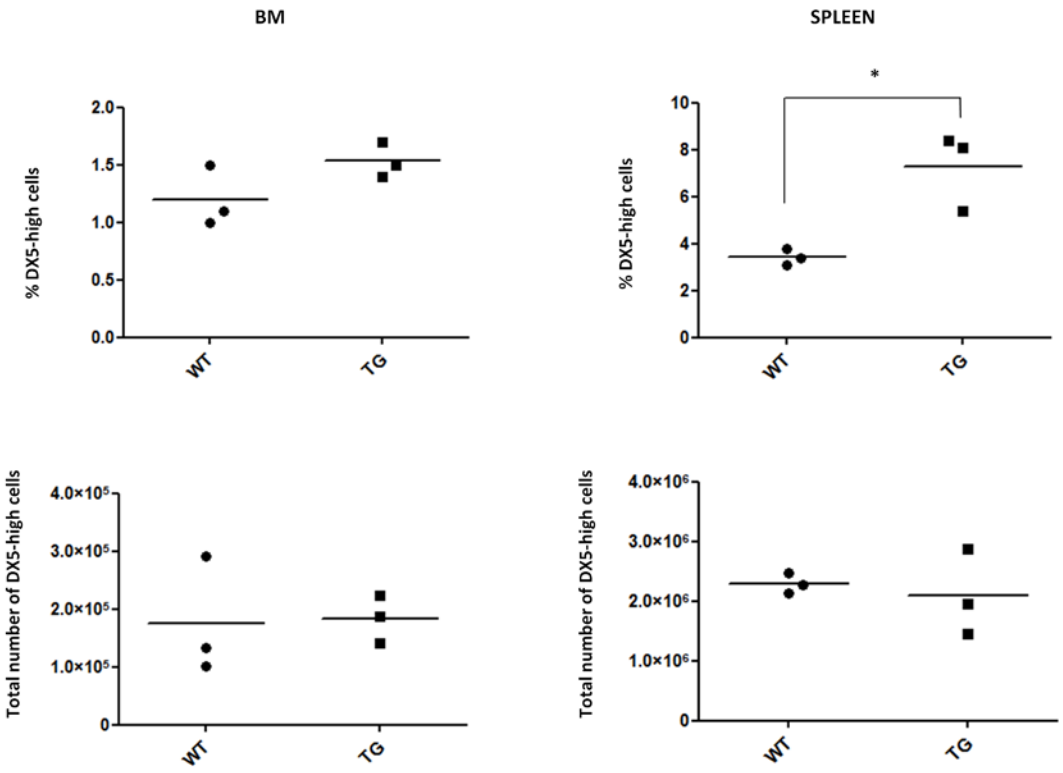


Fig. III.R.1.6. NK cells distribution in *CD69.BAC* transgenic BM and spleen. Percentages (*top*) or total number (*bottom*) of DX5^{high} cells in bone marrow (*left*) and spleen (*right*) in wild-type (WT) and *CD69.BAC* mice (TG) (*) $p<0.05$. Data representative of three experiments.

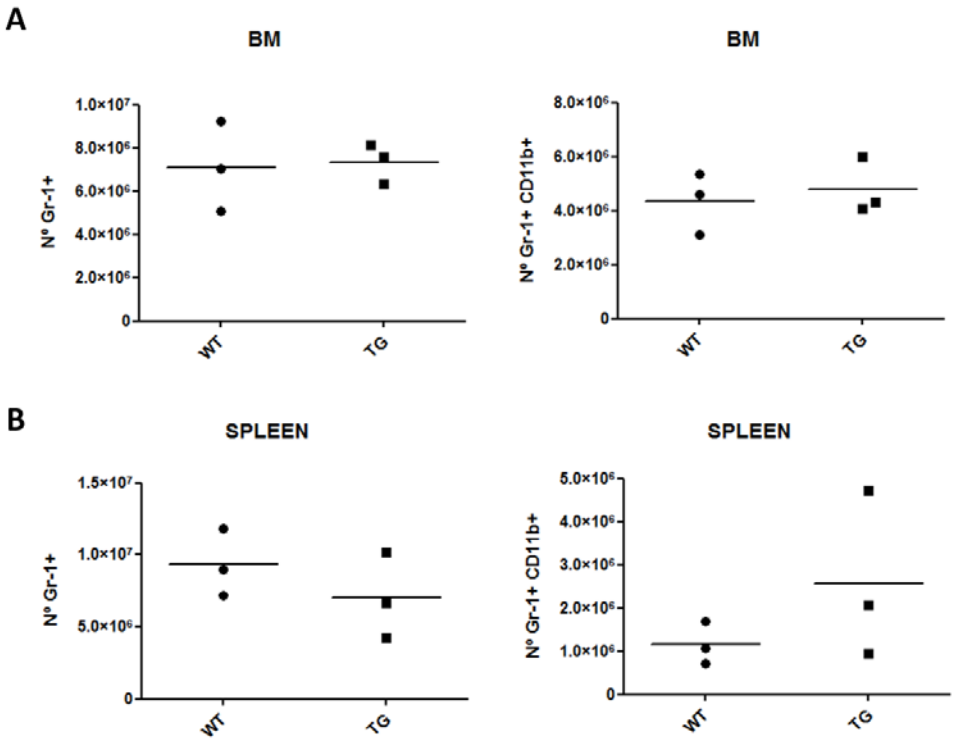


Fig. III.R.1.7. Granulocyte distribution in *CD69.BAC* transgenic lymphoid tissues. Total number of Gr-1+ (*left*), and Gr-1+ CD11b+ (*right*) cells were analyzed by flow cytometry in BM (A) and spleen (B) of wild-type (WT) and *CD69.BAC* (TG) mice. All differences were not significant. Data are representative of three experiments.

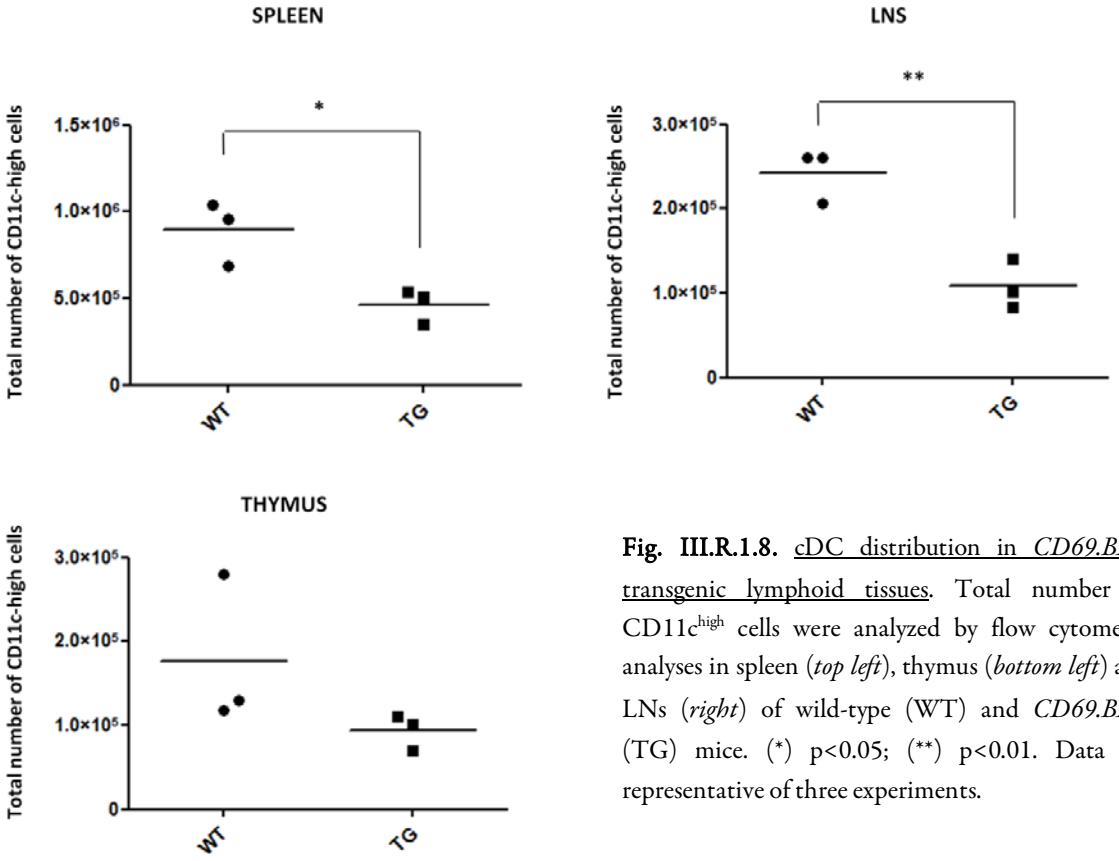


Fig. III.R.1.8. cDC distribution in *CD69.BAC* transgenic lymphoid tissues. Total number of CD11c^{high} cells were analyzed by flow cytometry analyses in spleen (*top left*), thymus (*bottom left*) and LNs (*right*) of wild-type (WT) and *CD69.BAC* (TG) mice. (*) $p<0.05$; (**) $p<0.01$. Data are representative of three experiments.

Granulocytes neither present differences in numbers in bone marrow and spleen (Figure III.R.1.7). Moreover, this population is augmented in percentage in blood (not shown); however, this increase might be caused by a reduction in lymphocyte levels.

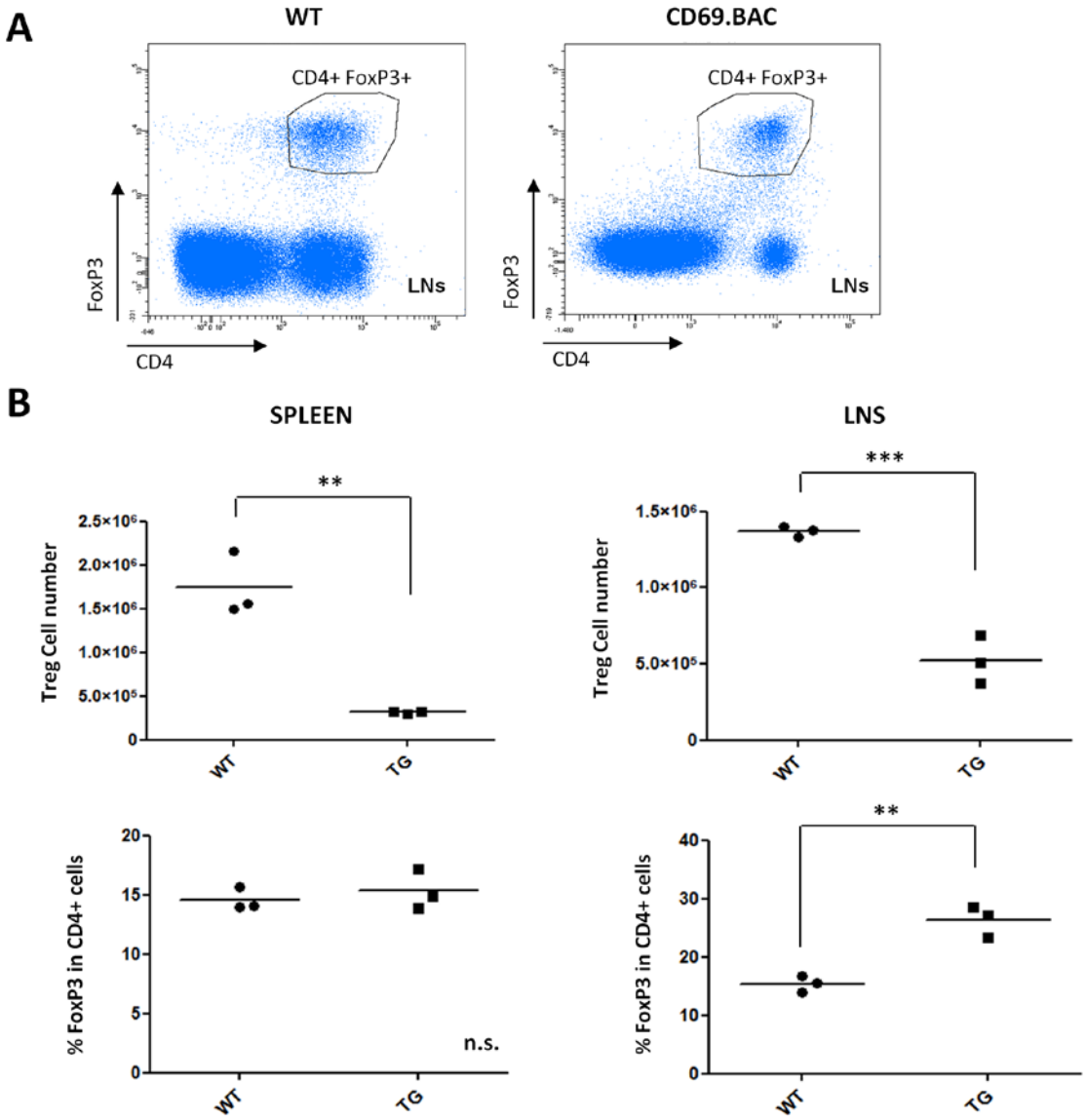


Fig. III.R.1.9. Treg distribution in *CD69.BAC* transgenic lymphoid tissues. A) CD4+ FoxP3+ populations from spleen and LNs were gated as indicated in wild-type (WT) and *CD69.BAC* (CD69.BAC) mice to analyze percentages and numbers showed in *B* and *C* (only plots of LNs are shown). B, C) Flow cytometry analyses of Treg population in spleen (*left*) and LNs (*right*) of wild-type (WT) and *CD69.BAC* (TG) mice. (B) *Top*, total count of CD4+ FoxP3+ cells; *bottom*, percentage of FoxP3+ in CD4-positive cells. (**) $p<0.01$; (***) $p<0.001$; (n.s.) non-significant. Data are representative of two experiments.

Regulatory T cells (Treg) distribution

Treg function has been established to be controlled by S1P1 expression (306), presenting high suppressive activity when the Treg cell down-regulates S1P1 receptor. S1P1 is also required for their proper development and egress from thymus.

Thus, we analyzed then Treg populations in TG mice, and found that CD4+ FoxP3+ population (known as "naturally-occurring" Tregs in contrast to Tregs induced in vitro (308)), is decreased in LN and spleen (Fig. III.R.1.9), similarly to CD4+ T cell population. However, when looking at the percentage of Tregs within CD4+ T cells, we observed that it was increased in lymph nodes of the tg, but not in the spleen. As CD69 has been related to regulatory populations in tumor (105) and infection (95) models, we studied relations between different markers of Tregs. Remarkably, CD4+ FoxP3+ CD69+ population is similar in spleen and LNs of tg and WT mice (Fig. III.R.1.9).

Altered CD44 pattern expression

In preliminary cytometric analyses, we found an augmented expression of the adhesion molecule CD44 in several lymphoid tissues of *CD69.BAC* mice (data not shown). As a deficient generation of memory Th2 cells was observed in BM of *CD69.KO.2* mice (121), we analyzed CD44 expression in CD3+ CD4+ cells of bone marrow, as the majority of memory T cells in BM express high levels of CD44 (309). We observed significantly reduced CD44 percentage in CD4+ T cell population, and significantly reduced expression (lower MFI) in CD3+ CD4+ cells (Fig. III.R.10) of *CD69.BAC* mice. The functional significance of this observation remains to be further studied.

To summarize, CD69 overexpressing mice show important alterations in leukocyte distribution, especially in B and T cells and also in CD11c^{high} dendritic cells

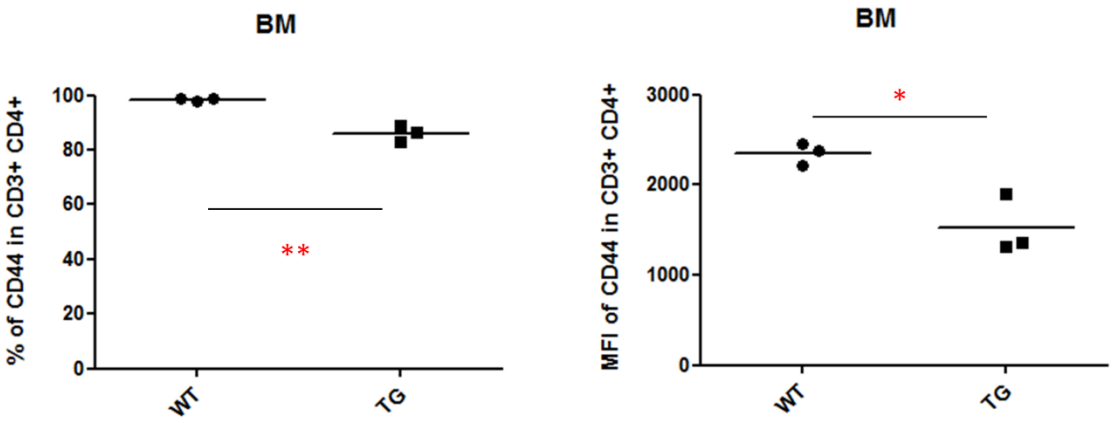


Fig. III.R.1.10. CD44 expression in CD4+ T cells from bone marrow in *CD69.BAC* transgenic mice. A) Percentage of CD44+ in CD3+ CD4+ cells. B) Mean fluorescence intensity (MFI) of CD44 in CD3+ CD4+ cells of WT and tg mice. (*) $p<0.05$; (**) $p<0.01$; (n.s.) non-significant. Data are representative of two experiments.

2. INCREASED PRESENCE OF REGULATORY T CELLS IN SPLEEN OF CD69 -/- MICE

In the previous chapter, it was shown that CD69-overexpressing TG mice line presented altered patterns of leukocyte distribution. Considering those results, *CD69.KO.1* mice were examined further, despite the fact that characterization studies have not found major differences in their leukocyte populations (89). As Treg populations were not studied in those works, we analyzed Treg proportions in WT and KO mice.

T_{regs} are augmented in CD69 knock-out spleen

Spleens from the C57BL/6 and BALB/c mouse strains were analyzed for Treg proportions. CD4+ FoxP3+ cells (Tregs). This population appears significantly increased in CD69 -/- splenocytes of both mouse strains, either considering its percentage among the total spleen cell numbers or among CD4+ T cells (Fig. III.R.2.1 for C57BL/6 and Fig. III.R.2.2 for BALB/c).

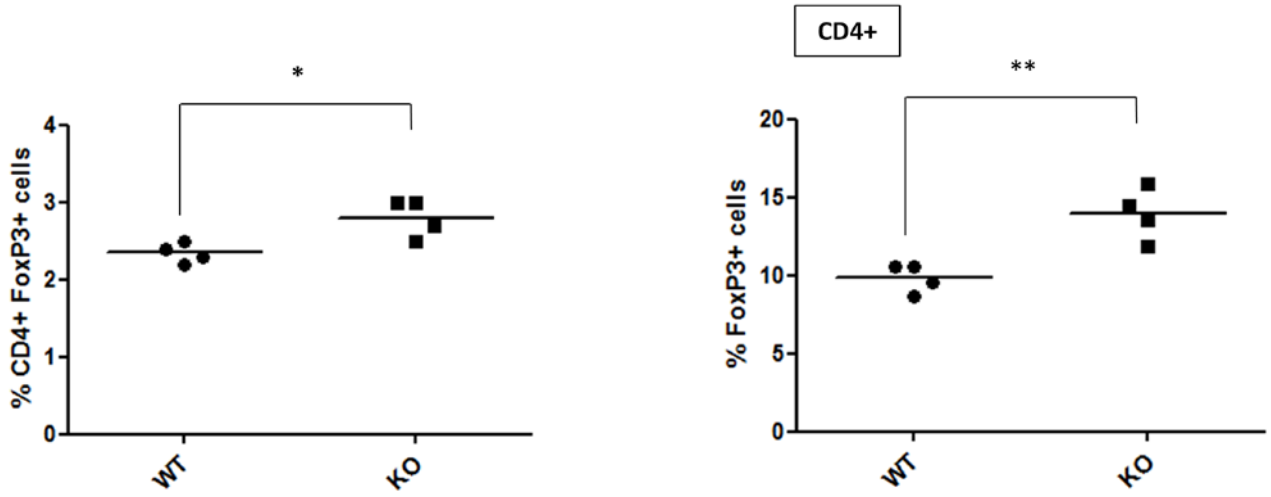


Fig. III.R.2.1. CD4+ FoxP3+ Treg population is increased in the *CD69.KO.1* (C57BL/6) spleen. Flow cytometry analyses of the splenic Treg population of wild-type (WT) and *CD69KO1* (KO) mice. *Left,* percentage of CD4+ FoxP3+ within total spleen cells. *Right*, percentage of FoxP3 positive cells within CD4+ population. (*) $p<0.05$; (**) $p<0.01$. Data representative of two

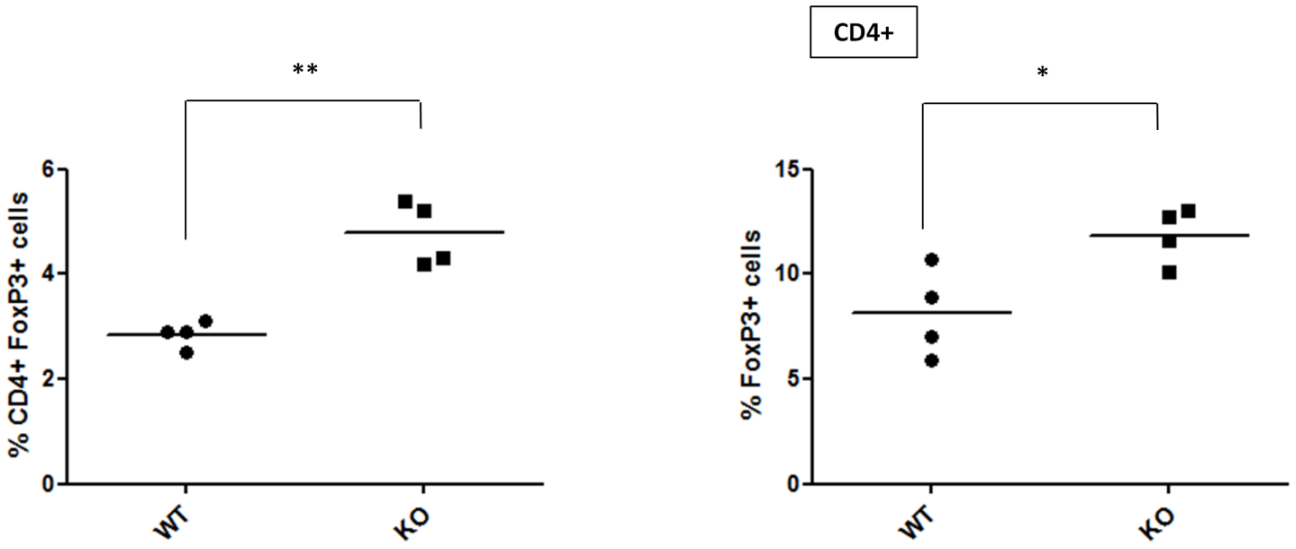


Fig. III.R.2.2. CD4+ FoxP3+ Treg population is increased in *CD69.KO.1* (BALB/c) spleen. Flow cytometry analyses of the splenic Treg population of wild-type (WT) and *CD69KO1* (KO) mice. *Left,* percentage of CD4+ FoxP3+ within total spleen cells. *Right*, percentage of FoxP3+ cells within CD4+ population. (*) p<0.05; (**) p<0.01. Data representative of two experiments.

Natural T_{regs} (CD4+ FoxP3+ induced in thymus) usually express CD25 receptor (310). Consistently, an increase in CD4+ CD25+ percentages is also observed in the BALB/c mouse strain (but not significantly in the C57BL/6) (Fig. III.R.2.3). Differences in the whole CD4+ population are not significant, suggesting that distribution or development of Tregs are more sensitive to the absence of CD69 than the ones of the entire CD4+ population (Fig. III.2.4).

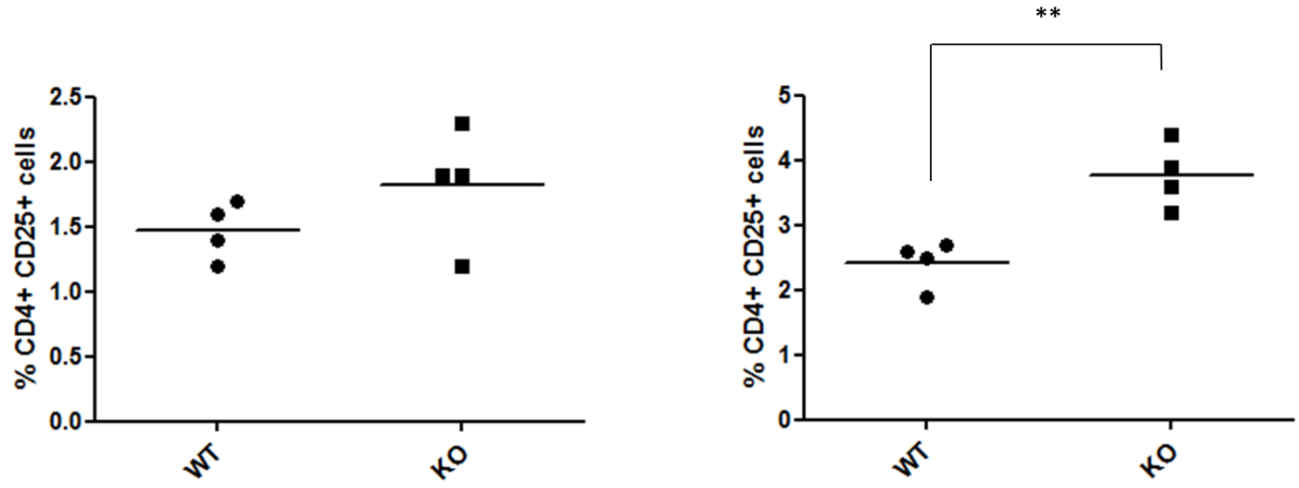


Fig. III.R.2.3. CD4+ CD25+ population increase in the spleen of *CD69.KO.1* and WT mice. Percentages of the splenic CD4+ CD25+ population in C57BL/6 mouse strain (*left*) and BALB/c (*right*). (**) p<0.01. Data representative of two experiments.

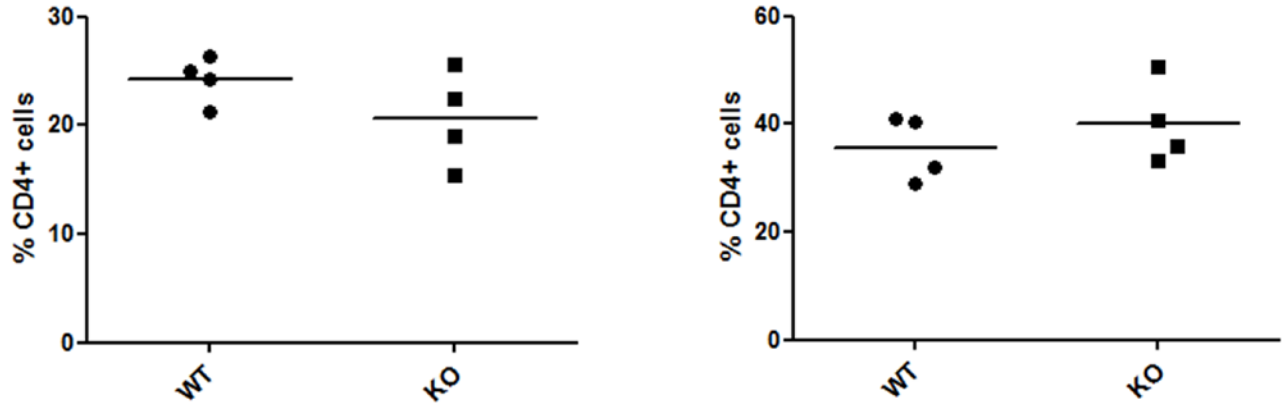
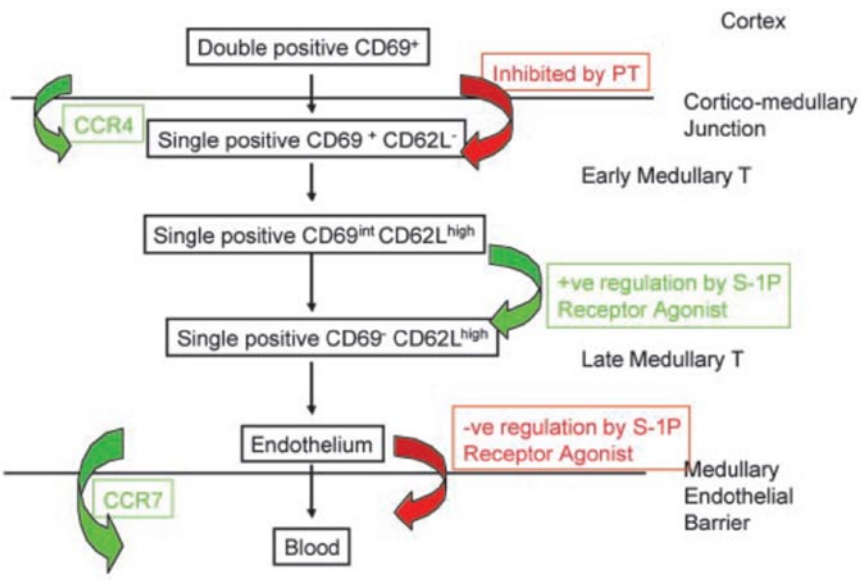


Fig. III.R.2.4. Percentage of CD4+ population in splenocytes of *CD69.KO.1* and WT mice. *Left*, percentages for C57BL/6 mouse strain; *right*, percentage for BALB/c. Data representative of two experiments.

DISCUSSION

In this chapter we studied the leukocyte distribution of *CD69.BAC* mice, which overexpresses CD69 constitutively in most leukocytes and carry high copy number of CD69 BAC transgene, in order to check for altered distribution of T lymphocytes, as previously described in models of mice overexpressing CD69 (110, 111). Indeed, we observed increased numbers of SP T cells in thymus and an important reduction in numbers of T lymphocytes in spleen and LNs, and reduced percentage in blood, agreeing with the previous data (110, 111). The results in TG mice are also in agreement with altered leukocyte distribution in CD69 -/- mice (96, 120). CD69KO2 mice exhibited an enhanced thymocyte egress (120). In this work, the CD69 function in thymocytes is suggested to be delaying the thymocyte egress for several hours, leading to the continuously elevated numbers of SP cells, which we also found in the thymus of *CD69.BAC* mice. The data above support the need of CD69 down-regulation to exit thymus attributed to the resulting S1P1 up-regulation, which allows to respond by chemotaxis to increased concentration of S1P outside the thymus (120, 300, 301) (Fig. III.D.1)

In the periphery, CD69 has also been observed as a regulator of lymphocyte trafficking under stimulation (112). This effect is shown in T and B lymphocytes and it also occurs by regulating S1P1 expression. This interaction between CD69 and S1P1 has been documented to be produced by direct contact in T cells (151), and in other leukocyte subpopulations, as in skin DCs (294), where their migration from skin to LNs



Rosen et al. , PNAS (2003)

Fig. III.D.1. Sequence of S1P receptor-regulated events in thymic maturation and egress. Double positive CD69pos T cells are found in the cortex, whereas SP CD69pos T cells are found in the medulla except in the presence of PTX when SP CD69pos T cells remain in the cortex. An S1P receptor activation-independent step results in generation of a population of CD69int SP T cells, which then undergo rapid maturation to CD69neg SP cells induced by S1P receptor agonism. The egress of all mature cells is then rapidly negatively regulated by S1P receptor activation, inhibiting the CCR7-dependent appearance of mature cells in secondary lymphoid organs.

is accelerated in CD69KO1 mice than in WT counterparts. Even if we did not stimulate cells, our results in *CD69.BAC* mice still showing a significant lesser presence of conventional DCs in spleen and LNs also point towards the same direction. This hyphothesis needs to be further studied considering transference experiments and analysis of different subpopulations of DCs.

The altered distribution of B cells appreciated in TG mice correlates with the S1P1 -/- phenotypes in B cells previously reported, as they showed mature B cell increase in BM (303, 304), in addition to reduced numbers of B cells in spleen and blood and similar quantities in LNs (304). Moreover,, it was reported an enhanced expression of CD69 in the marginal zone (MZ) B cells in S1P1 B-restricted KO (311), which associates again CD69 and S1P1 altered expression in models with altered distribution of leukocytes.

In this chapter we report for the first time an altered distribution of the regulatory T cell population (CD4+ FoxP3+) in an *in vivo* model of overexpression of CD69. We observed a reduction in total Treg numbers in spleen and LNs of *CD69.BAC* mice respect to WT littermates, while proportion of FoxP3+ among CD4+ T population remains unchanged in spleen and increases in LNs, being the balance between Treg and non-Treg considerably enhanced in periphery. Furthermore, *CD69KO1* mice exhibited augmented percentages of CD4+ FoxP3+ cells among the total splenocytes and of FoxP3+ among the CD4+ T population. These data are in agreement with previous studies which showed a reduced proportion of FoxP3+ cells among CD4+ T population in LNs and similar in spleen of CD69KO2 compared to WT mice (106), and with the fact that S1P1 KO mice in T cells, which exhibited augmented percentages of FoxP3+ cells within CD4+ T cells in LNs (306). The results we obtained in CD69KO1 correlated in part with these studies, as we appreciated increased percentages of Treg population among the total splenocytes. However, the proportions of FoxP3+ cells within CD4+ T splenocytes are also elevated, suggesting the distribution of Tregs and non-regulatory T cells is differently regulated by CD69, but this needs further evidences to be confirmed. As Treg development, function and distribution have been observed to be controlled by S1P1 (306), it is necessary to further analyze the functional characteristics of Treg population in *CD69.BAC* and *CD69KO1* mouse lines.

The role of CD69 in leukocyte migration mediated by other S1P receptors distinct from S1P1 has been studied. *In vitro* assays have not shown interaction of S1P5 and CD69 nor CD69 influences S1P5-mediated migration to S1P gradients (312). As S1P5 proper expression is required for effective exit of NK cells from bone marrow to circulation (312, 313), it is proposed that CD69 does not participate in NK cell migration

(312). Our observations of non-altered NK cell numbers in BM and spleen of CD69-overexpressing mice correlate with that hypothesis. Moreover, CD69 and S1P3 expressions seem not to be related (151, 294). Nevertheless, other S1P receptors have been defined as relevant for specific leukocyte subpopulations that express CD69, as S1P4 for plasmacytoid DCs (314) and for B cells (303, 304).

Despite the fact that more analyses are required, like S1P1 mRNA and protein expression analysis, and distribution studies of challenged CD69.BAC mice and transference of its cells, the effects observed in CD69-overexpressing mice support the previous data and suggest a major role of CD69 in leukocyte distribution. Whether these studies would unveil CD69 additional roles in immune function needs further research work. As the altered distribution was observed in various types of leukocytes, we consider the BAC-in mice *CD69.BAC* we have generated as a good model to continue the functional studies of CD69.

DISCUSIÓN

REGULACIÓN DE LA EXPRESIÓN DE CD69 Y SUS EFECTOS FUNCIONALES

Los objetivos de esta tesis eran profundizar en el estudio de la regulación génica de CD69, y como afectaba a su expresión y su función en el sistema inmune.

A) REGULACIÓN TRANSCRIPCIONAL DE CD69

Estudios previos han establecido el promotor, y CNS1, CNS2, CNS3 y CNS4 como elementos reguladores en *cis* de la transcripción de CD69 (46, 142, 315) y como elementos reguladores *trans* con unión al promotor, NFκB (315) y AP-1 (145).

Para profundizar en el estudio de la regulación génica de CD69, se han empleado diferentes estudios *in silico* y funcionales, que han permitido confirmar la función reguladora de los elementos *cis* anteriormente descritos y establecer una nueva por parte del Intrón I de CD69, en el que primeramente se estudió la accesibilidad de su parte de cromatina. La principal conclusión es la inducibilidad de la apertura de la cromatina en esta región, concretamente en la zona de hipersensibilidad HSa, siendo además prácticamente específica de líneas hematopoyéticas, salvo por una ligera apertura observada en la células HUVEC. Existen evidencias adicionales de la expresión de RNA de CD69 en células CD105+ endoteliales (234-236), de manera que son necesarios más estudios sobre la expresión y la función de CD69 en endotelio. La especificidad para el linaje hematopoyético de accesibilidad de la cromatina de CD69 también se ha observado para el promotor, CNS1 y CNS2. Estos datos sugieren una apertura de cromatina específica de locus y de tejido.

Mediante las mismas herramientas empleadas con el Intrón I, hemos definido una región mínima responsable de la inducción por activación de TCR y PKC en la zona conservada no codificante CNS2, compuesta por los dominios A y B. En esta región el papel de RUNX1 parece el más relevante a la vista de los resultados en la expresión de reporteros de luciferasa y el silenciamiento de su RNA, ya que aunque el dominio donde se encuentra por sí solo no es capaz de inducir la actividad transcripcional del promotor, la eliminación y la mutación de su sitio de unión elimina en una cantidad notable dicha actividad, mayor que las mutaciones en otros TFBS. Estos datos se apoyan en los estudios sobre el ratón *knock-in* condicional de

RUNX1 en células CD4+, donde se aprecia una disminución significativa de la expresión de CD69 en timocitos (238). La falta de cualquier actividad inductora del vector que contiene el promotor dirigido únicamente por el dominio B indica que la función de RUNX1 en dicho dominio necesita de la cooperación de factores de transcripción que se unan al dominio A. Aunque no se descarta el efecto de RUNX1 en otras zonas reguladoras en *cis,* ya que presenta un sitio de unión conservado en CNS3 (Capítulo I), según los resultados obtenidos tras el silenciamiento de su RNA, la relevancia aportada a CNS2 tanto en apertura de cromatina, como en marcas de histonas, como en la unión de factores de transcripción por la plataforma ENCODE (http://encodeproject.org/ENCODE), hace bastante probable que su acción mayoritaria en regulación de CD69 ocurra en su sitio en CNS2. El hecho de que RUNX no figure como factor de transcripción que se una a CNS2 en los datos de ENCODE no descarta su posible función, debido a que los datos del consorcio no recogen las inmunoprecipitaciones de RUNX1.

La cooperación entre factores de transcripción está ampliamente estudiada, habiéndose definido varios modelos funcionales (26). El modelo que más encaja con el funcionamiento del núcleo formado por los dominios A y B de CNS2, es el conocido como *billboard* en inglés, que consiste en que ninguno de los factores que se unen al núcleo es imprescindible, actúan de manera cooperativa, y sus secuencias de unión sí son necesarias. Otro hecho importante es que en este núcleo varios sitios de unión de los factores solapan, pudiendo inhibirse unos a otros y reflejando la complejidad de la actividad regulatoria. Además, la cooperación ente factores suele ocurrir formando complejos multiproteicos, de tal forma que estos complejos pueden modular su regulación mediante desplazamientos e intercambios de subunidades en su unión al DNA específicamente en función del tipo celular, fase de desarrollo, etc. Se ha observado la cooperación entre RUNX y Elk-1 (205), y una regulación opuesta en células inmunes entre la expresión de Elk-1 y GABPA (210). En nuestro caso la posible interacción directa de RUNX1 con estos 2 factores no es suficiente para inducir la transcripción de CD69 en la línea T CD4+ Jurkat, si no que se necesita la cooperación de un elemento del dominio A. El candidato más probable para hacer inducir la función de RUNX es SRF, del que a pesar de que no se han descrito interacciones entre ellos, los datos de unión de factores de transcripción obtenidos por ChIP-seq en ENCODE muestran una importante unión de SRF a CNS2 en células K562.

Como conclusión, cabe destacar que la regulación transcripcional estudiada en CD69 indica una especificidad de linaje celular, ya que normalmente en células no hematopoyéticas no se encuentra disponible la cromatina para ser accesible a regulación por factores de transcripción. Los elementos

promotor, CNS2 y, con alta probabilidad, RUNX, parecen los responsables principales de la actividad transcripcional en el linaje hematopoyético.

B) REGULACIÓN DE LA EXPRESIÓN DE CD69 EN MODELOS TRANSGÉNICOS CD69-BACin

El modelo de ratón transgénico (TG) cuyo vector insertado contenía el gen de detección hCD2 dirigido por el promotor, CNS1 y CNS2 clonados sin sus espacios intermedios (46), mostró una inhibición de la transcripción elevada y diferente entre linfocitos T y B. Este transgén carecía de posibles elementos reguladores intragénicos, las zonas no traducidas (UTR) y otros elementos *cis* distales, por ello se utilizó el modelo de transgénesis mediante BACs para que el entorno genómico del gen de CD69 en el transgén se pareciera lo máximo posible al entorno del gen endógeno. El objetivo era poder detectar una expresión regulada de la molécula para posteriormente poder eliminar zonas reguladoras del gen y analizar sus efectos. No obstante, al realizar la transgénesis de BACs de CD69 conteniendo el cDNA para GFP, se detectó una elevada expresión del transgén en la mayoría de las líneas hematopoyéticas de la líneas de ratón con alto número de copia del transgén (*CD69.BAC*) y en la línea celular no hematopoyética CHO; mientras que en el resto de líneas de ratón y en la línea T CD4+ Jurkat no se observó.

El hecho de que la transgénesis en baja copia no produjera expresión del transgén en líneas de ratón y la expresión en líneas celulares fuese dependiente de la línea, revela que la transgénesis de BAC no aporta todos los elementos y/o mecanismos necesarios para la correcta regulación de la expresión génica. Debido a que los 3 BACs empleados poseen los elementos *cis* intrón I, promotor, CNS1, CNS2, CNS3, CNS4 y otros posibles intra- e intergénicos, hace pensar poco probable que lo que falte sea un elemento *cis* en la regulación transcripcional. Sin embargo, caben otro tipo de fenómenos que puedan explicar la no-regulación en la transgénesis de BAC-CD69-GFP. Por un lado, la falta de otros elementos reguladores, quizá una región controladora de Locus (*Locus Control Region*, LCR), regiones que regulan la transcripción de varios genes situados a grandes distancias genómicas (291), puede que impidan su expresión. No obstante, el ratón *CD69.BAC* sí que expresa el transgén, desconociendo el mecanismo por el cual la presencia de un elevado número de copias de un gen activaría la transcripción. Una explicación más probable sería por la necesidad de la región génica de estar localizada en su sitio fisiológico, donde intervendrían aspectos como el plegamiento de la cromatina y la localización en lo que se conoce como el "territorio cromosómico" (287,

288, 316). Los datos obtenidos para la expresión del transgén en timo apoyan en parte esta idea, donde parece que para la expresión del transgén es necesaria la expresión previa de CD69 endógeno, cabiendo la posibilidad de que interviniese un fenómeno de preferencia alélica (317, 318). Este fenómeno no se aprecia en otros tipos celulares, ni siquiera en linfocitos B, sugiriendo que la regulación de CD69 en linfocitos T es específica y dependiente del desarrollo.

A pesar de la alta expresión tanto de GFP como de CD69 en el ratón *CD69.BAC*, se apreciaron patrones de expresión de las 2 moléculas exclusivos de cada tipo celular leucocitario, lo que sugiere que hay uno o varios mecanismos reguladores de tipo general que no funcionan correctamente en la línea con alto número de copia de transgén, pero sí que lo hacen mecanismos específicos de cada linaje leucocitario. La expresión del transgén en otros tipos celulares distintos de los hematopoyéticos está aún en estudio. Un hallazgo relevante en esta línea de ratón se refiere a la diferente expresión del transgén, es que las células de tipo linfoide y las células dendríticas convencionales presentan altos niveles de expresión, mientras que las de tipo mieloide, como los granulocitos maduros, son capaces de disminuir la expresión de CD69 a niveles parecidos a los del ratón WT. Se deduce que los granulocitos disminuyen la expresión del transgén debido a que sus precursores hematopoyéticos de médula ósea (c-kit+ Gr-1+) expresan CD69 a niveles más altos que los granulocitos maduros (aunque no tanto como los linfocitos maduros), frente a los precursores del ratón WT que no expresan CD69. Este hecho concordaría con que pudiera haber una diferente velocidad en la regulación de CD69, tanto a nivel transcripcional como de niveles de RNA o proteína, tal y como se ha reportado en distintos estudios al activar diferentes tipos celulares. En estos se observa que mientras que las líneas mieloides inducen más lentamente la expresión, siendo incluso más lenta que otras moléculas expresadas tras activación (74, 77, 78), en linfocitos la expresión es prácticamente instantánea, tanto en RNA como en proteína (revisado en (246)). Análisis más exhaustivos estudiando concentraciones de RNA y proteína tanto en el ratón TG como en el WT son necesarios para confirmar esta hipótesis y establecer si este fenómeno ocurre fondos genéticos no modificados. La diferente expresión de CD69 y GFP indica que ambas moléculas sufren regulaciones post-transcripcionales diferentes, lo cual no es extraño debido a la elevada estabilidad de la proteína GFP (296, 319).

Otro hecho importante observado en el ratón *CD69.BAC* es que la expresión de CD69 (pero no de GFP) está ligeramente modulada por la localización celular, siendo la mayor en LNs, timo y sangre, media en médula ósea y menor en bazo para los linfocitos. Para los granulocitos, la máxima expresión ocurre en médula ósea y la menor en sangre. Este tipo de regulación también ocurre en el ratón WT, sin embargo, el

número de células que expresan CD69 es mucho menor. Serían adecuados estudios sobre la cantidad de RNA y proteína intracelular en las células CD69– de ambas líneas de ratón para averiguar qué tipo(s) de regulación sufren los leucocitos CD69– de la línea WT.

En definitiva, aparte de los mecanismos transcripcionales explicados en el capítulo I se hipotetiza que intervienen otro tipo de mecanismos en la regulación de la expresión de CD69, como pueden ser la regulación de las concentraciones de mRNA y proteína. En cuanto a la regulación de RNA, existen evidencias de la regulación por el fragmento 3' transcrito pero no traducido (3' UTR), que confiere inestabilidad al mRNA de CD69 (146), y es diana de la degradación mediada por los micro-RNAs miR-130/301 (147) y miR-181 (148). En esta región existen otros sitios de unión a miRNAs conocidos (Fig. D.1), entre ellos miR-155, que ha sido asociado en varios trabajos a la activación de leucocitos (320-322). Además, CD69 también es regulado a nivel proteico por S1P1 (112, 149-151). El modelo propuesto consiste básicamente en que ante una señal que induzca la internalización de cualquiera de los dos receptores, éste se asociaría a una molécula del otro y se internalizarían ambos, pudiendo reciclarse y volver a salir, o bien, dirigirse a una vía de degradación. Por todo ello, son necesarios estudios adicionales sobre los niveles de RNA y proteína de CD69 y S1P1 en los modelos de ratón *CD69.BAC*, WT y *CD69KO1* para esclarecer los posibles mecanismos post-transcripcionales que intervienen en la regulación de CD69.

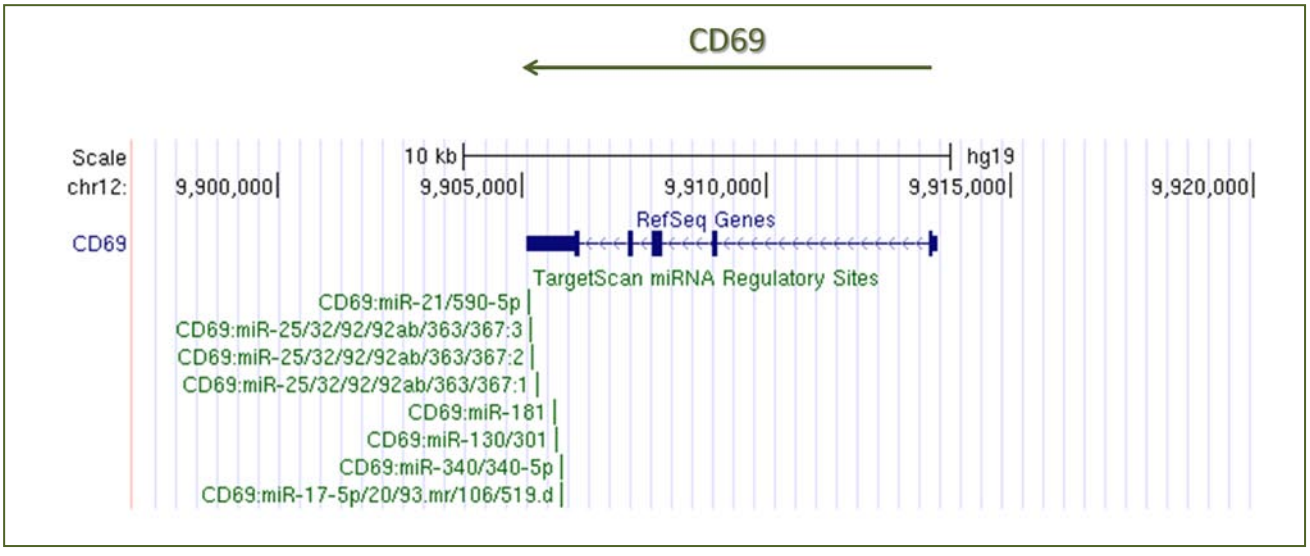


Fig. D.1 Micro-RNAs con secuencias de unión a la región 3' UTR de CD69.*Parte superior*, el gen de CD69 y la dirección de su cadena (+) están indicados con una flecha verde. *Parte inferior*, posición de las secuencias de unión al 3' UTR de CD69 de los miRNAs nombrados indicada con una línea vertical verde. Datos de la plataforma TargetScan extraídos del navegador de la UCSC.

C) ASPECTOS FUNCIONALES DE LA REGULACIÓN DE LA EXPRESIÓN DE CD69

En este trabajo hemos generado una línea transgénica BAC-in de CD69-GFP que expresa CD69 en prácticamente todos los tipos de leucocitos estudiados (*CD69.BAC*), mayoritariamente en niveles más elevados que en el ratón WT. Esta sobreexpresión ha producido una distribución alterada de algunas poblaciones leucocitarias: linfocitos T (y dentro de ellos también en células Tregs), linfocitos B y en células dendríticas convencionales ($CD11c^{high}$).

Respecto a los linfocitos T, se ha observado un aumento en número de timocitos SP (CD4+ CD8- y CD4- CD8+) en el timo, como se describió previamente en los modelos TGs convencionales con alta copia del cDNA de CD69 (110, 111). Estos resultados están de acuerdo con la aumentada velocidad de salida de los timocitos en el ratón CD69KO2 (120), donde se sugiere como función de CD69 la de retener los timocitos SP en el timo antes de su salida a linfa. Además, también se observó un incremento en el número de células en los bazos y un aumento del número de células T en el peritoneo del ratón CD69KO1 (96), lo que concuerda con la reducida presencia de células T que hemos encontrado en periferia en el ratón *CD69.BAC*.

También se apreció una distribución de las células B diferente en bazo y LNs del ratón TG, lo que coincide con los resultados obtenidos en los modelos de ratón con deficiencia genética en S1P1 específica de células B (303, 304), molécula con la que se ha relacionado inversamente su expresión con la de CD69 en la regulación de la migración de linfocitos T y B (112) y células dendríticas (294). De hecho, en estos modelos de células B se ha observado una aumento de la expresión de CD69 en células B de la zona marginal de bazo (311).

Por otro lado, en esta tesis se expone por primera vez una distribución alterada en células Tregs en el modelo de sobreexpresión *in vivo* de CD69, donde se observa una disminución de la población de Tregs CD4+ FoxP3+ en bazo y LNs, al igual que la población entera T CD4+. Sin embargo, esta disminución no significa una reducción de la proporción de células FoxP3+ en la población CD4+, sino que es similar a la del WT en bazo e incluso mayor en los LNs. Esto conlleva un aumento considerable de la proporción entre células Tregs y T no reguladoras, lo que podría producir diferentes respuestas en distintos modelos de desafío inmune, cuestión pendiente para futuros estudios. Estos datos concuerdan con los análisis de proporciones de células FoxP3+ en la población CD4+, en la línea de ratón CD69KO2, donde la proporción es menor que en la línea WT en LNs y similar en bazo (106); y con los datos del modelo S1P1 KO en células T donde se observa un aumento de FoxP3+ en las células CD4+ de LNs (306). Los

resultados obtenidos del ratón CD69KO1 se corresponden en parte con los anteriores, ya que se observa una proporción aumentada de Tregs en el total del bazo; sin embargo, el porcentaje de células FoxP3+ en el total de las CD4+ también aumenta, sugiriendo que la regulación de la distribución entre Tregs y las células CD4+ no Tregs no funciona en la misma medida. Como se ha observado una influencia entre los niveles de S1P1 y la función y el desarrollo de las células Tregs (306) es importante analizar la funcionalidad de las células CD4+ FoxP3+ en los modelos *CD69.BAC* y *CD69KO1*.

Un dato a destacar es que las subpoblaciones de leucocitos con patrones de migración alterados observados en la línea *CD69.BAC* son los que presentan mayor expresión de CD69 y del transgén (Capítulo II). Podría plantearse por tanto, que el correcto tráfico leucocitario requiere unos niveles adecuados de CD69. Curiosamente, también se produce una elevada expresión del transgén en células progenitoras (c-kit+), donde no han apreciado diferencias significativas en su distribución. El hecho de que precursores hematopoyéticos (CD34+) también expresen S1P1 y su migración se afecte con la variación de su expresión (295), hace interesante un estudio en mayor profundidad de estas células en los modelos *CD69.BAC* y *CD69KO1*.

En resumen, la distribución leucocitaria se ve alterada en el modelo *in vivo* de sobreexpresión de CD69 en leucocitos. Futuros análisis de transferencia de células, medición de los niveles de S1P1 en RNA y proteína, ensayos de supresión de Tregs, desafío inmune del ratón *CD69.BAC*, etc. pueden aportar más evidencias sobre el posible papel de CD69 en la migración de leucocitos, a través o no de su interacción con la molécula S1P1.

Las herramientas empleadas, tanto experimentales como teóricas han abierto diferentes vías del estudio de la regulación de la expresión y la función de CD69, como el estudio de su regulación post-transcripcional o análisis de su efecto en la migración de leucocitos. Estas vías han quedado abiertas para futuras pruebas experimentales que ayuden a esclarecer los puntos expuestos anteriormente.

CONCLUSIONES

- REGULACIÓN TRANSCRIPCIONAL DE CD69

1. Los estudios *in silico* y funcionales realizados en el Capítulo I sugieren la controlada regulación transcripcional de CD69 mediante las zonas conservadas no codificantes (CNSs) y la región HSa del Intrón I.

2. Las señales de cromatina activa y la unión de factores de transcripción aportadas por datos predictivos y experimentales, son específicas de linajes celulares, casi exclusivamente de células de origen hematopoyético.

3. La secuencia núcleo (dominios A y B) que contiene los sitios de unión a RUNX y SRF en CNS2, parece ser el máximo responsable, junto con el promotor, de la regulación transcripcional de CD69. Esta idea se basa en que por sí sola esta zona produce una amplificación similar al CNS2 completo y en su ausencia, desaparece toda la actividad. También está fundamentada en los datos de ensayos de luciferasa con otros CNSs previos y en los datos de alta capacidad de unión de factores de transcripción (Consorcio ENCODE) comparados con los demás CNSs.

4. Los datos de eliminación del dominio B y de silenciamiento de RUNX1 sugieren que este factor es el más relevante en la función reguladora de la secuencia núcleo de CNS2. Este factor necesita de la acción de uno o más factores que se unan al dominio A, siendo SRF un candidato para actuar sobre la función de RUNX1, debido los datos de alta unión en líneas celulares hematopoyéticas a CNS2 (ENCODE).

5. Los estudios predictivos de la unión de factores de transcripción se consideran fiables y adecuados para desarrollar estudios sobre regulación génica, ya que han definido sitios de unión importantes para la actividad transcripcional de CNS2.

- CONTROL DE LA EXPRESIÓN DE CD69 EN LA TRANSGÉNESIS DE BACS

6. La transgénesis de BACS carece de los elementos y/o mecanismos necesarios para la correcta regulación de la expresión de CD69, ya que el transgén no se expresa cuando se integra en baja copia. El elevado número de transgénesis realizadas tanto en ratón como en líneas celulares, hace que la

probabilidad de una falta de expresión debido al lugar de inserción del transgén en el genoma sea baja.

7. Un elevado número de copias de BAC-CD69 produce una expresión constitutiva pero específica en cada tipo de leucocito, siendo muy elevada en el linaje linfoide (linfocitos T y B) y similar a la endógena en células mieloides (granulocitos).

8. La línea de ratón que posee un alto número de copias de BAC, *CD69.BAC*, presenta alteraciones importantes en la distribución de leucocitos:
    - Los linfocitos T presentan una retención en timo y menor cantidad en periferia.
    - Los linfocitos B se encuentran reducidos en en bazo y sangre.
    - Las células dendríticas convencionales están reducidas en número en bazo, ganglios linfáticos y en timo.
    - Las células T reguladoras se encuentran disminuidas en número, pero no en proporción respecto a la población T CD4+, en órganos linfoides secundarios.
    - No se han apreciado alteraciones en la distribución de los granulocitos y las células NK.

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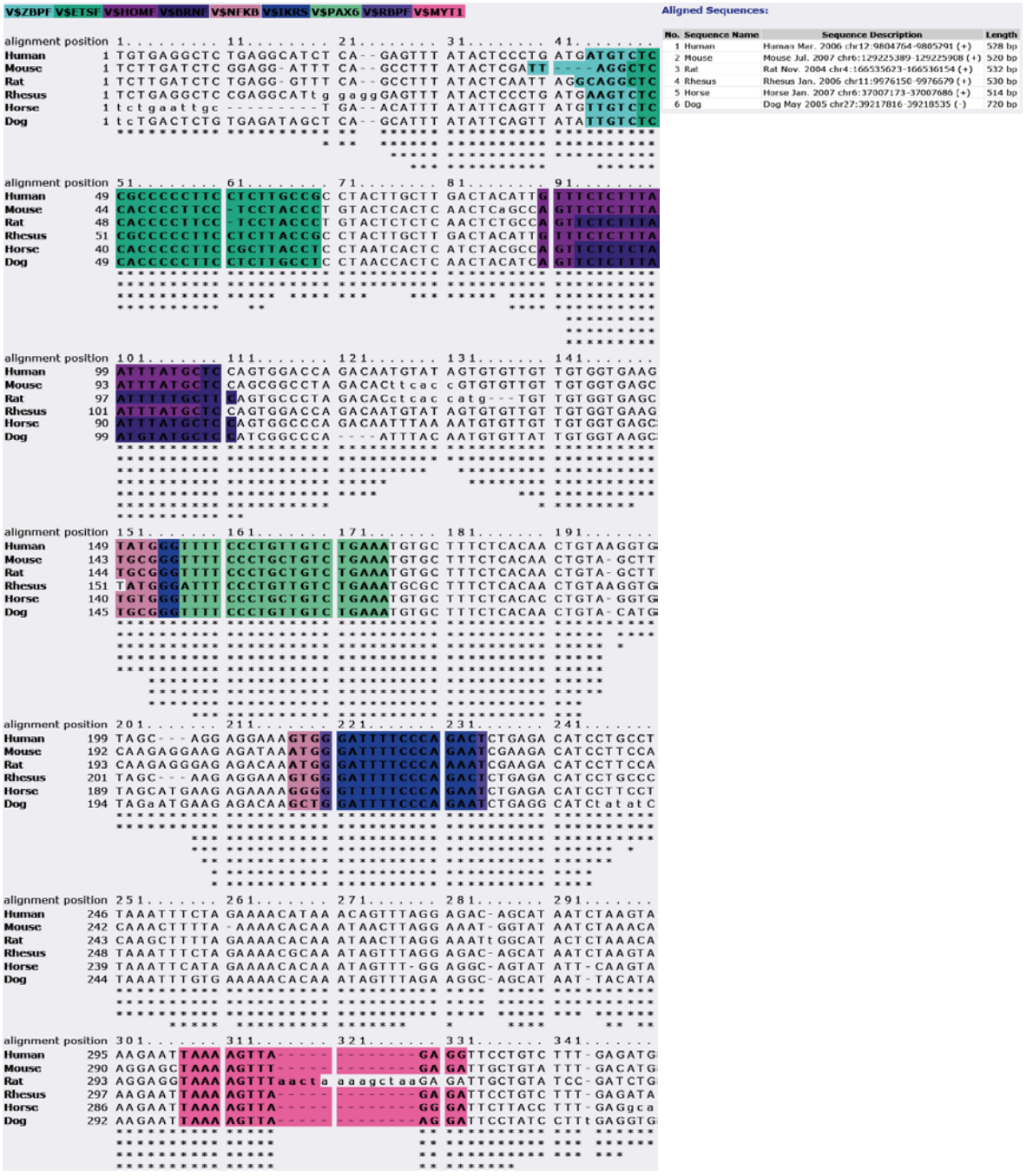
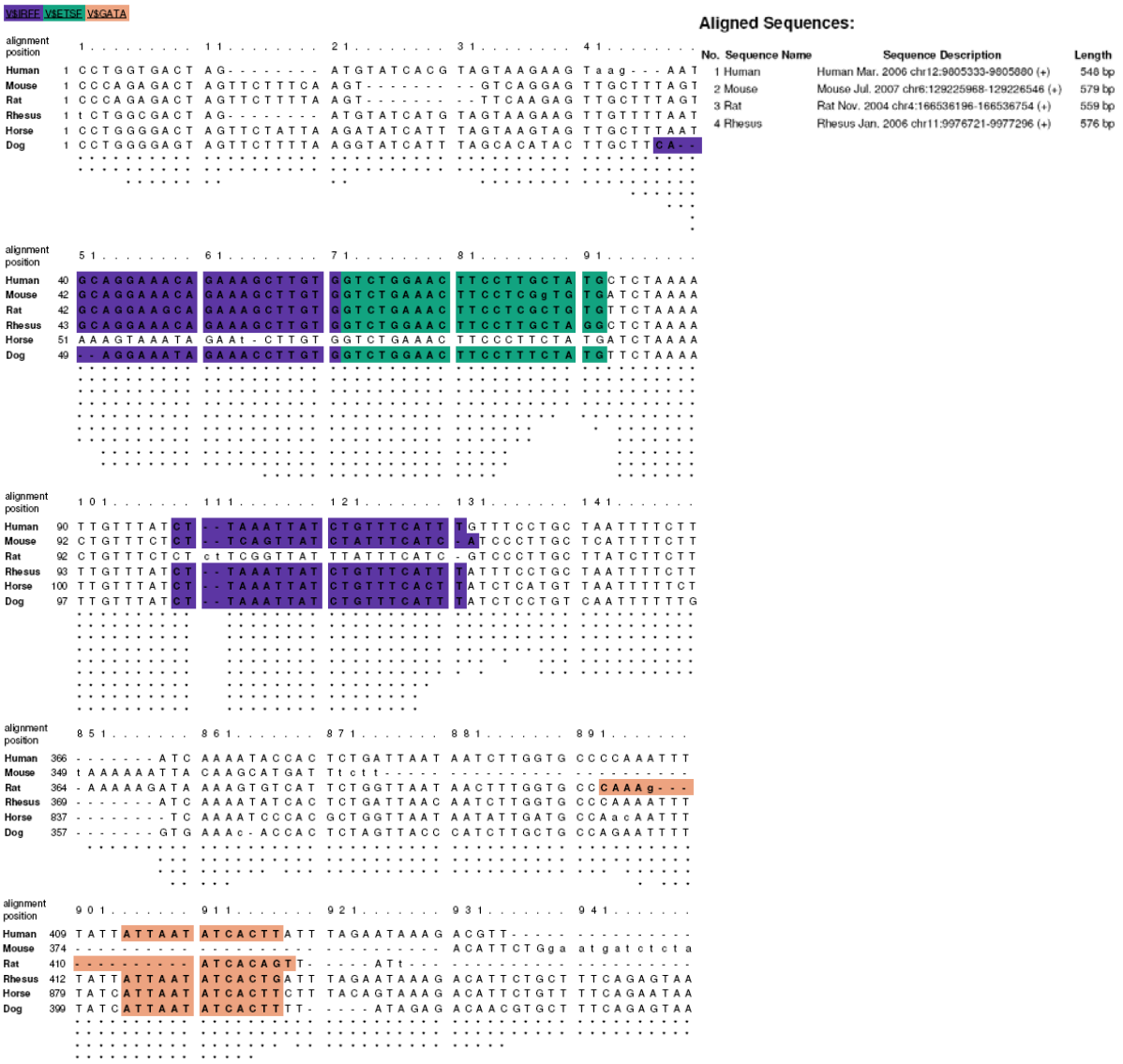
APÉNDICES

APÉNDICE I / APPENDIX I

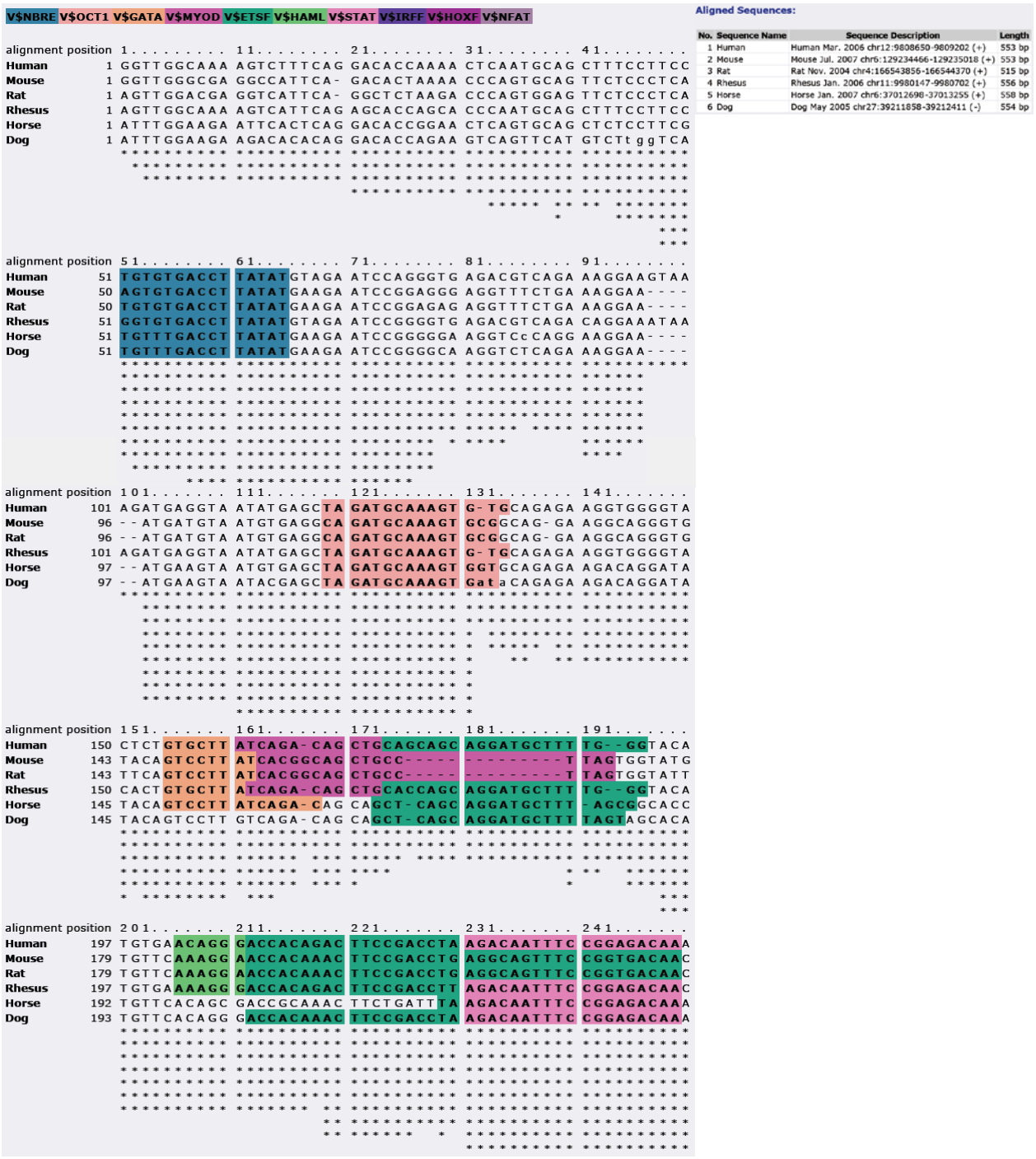
Supplementary Figure 1. Direct results of *Genomatix DiAlign Plus TF* for CD69 CNS1. Aligned sequences for 6 mammals are displayed: human, mouse, rat, rhesus, horse and dog. Common transcription factor binding sites (TFBS) are different colored according to the TF. *Top*, legend of TF families. For more information about TF, see Chapter I.



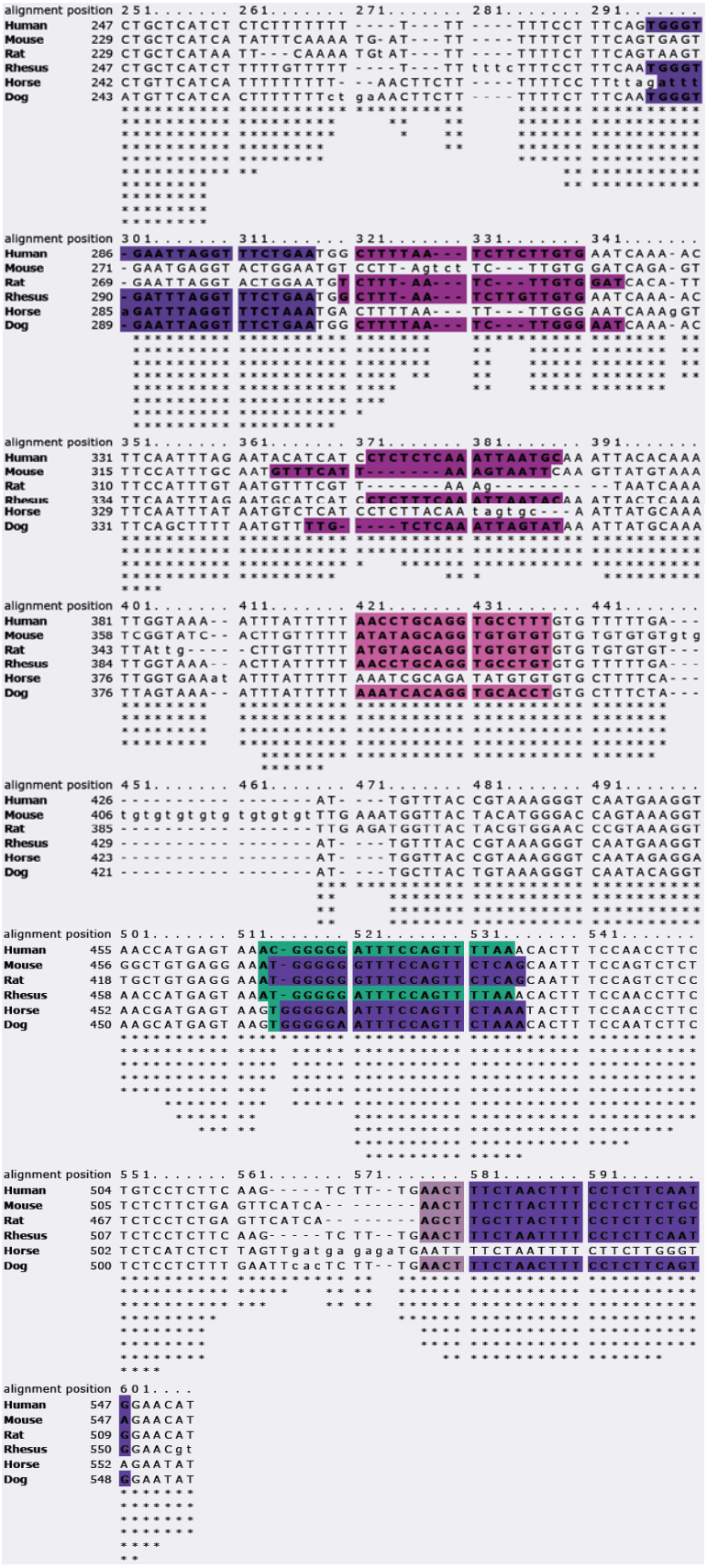
Supplementary Figure 1. (Cont.)



Supplementary Figure 2. Direct results of *Genomatix DiAlign Plus TF* for CNS2.



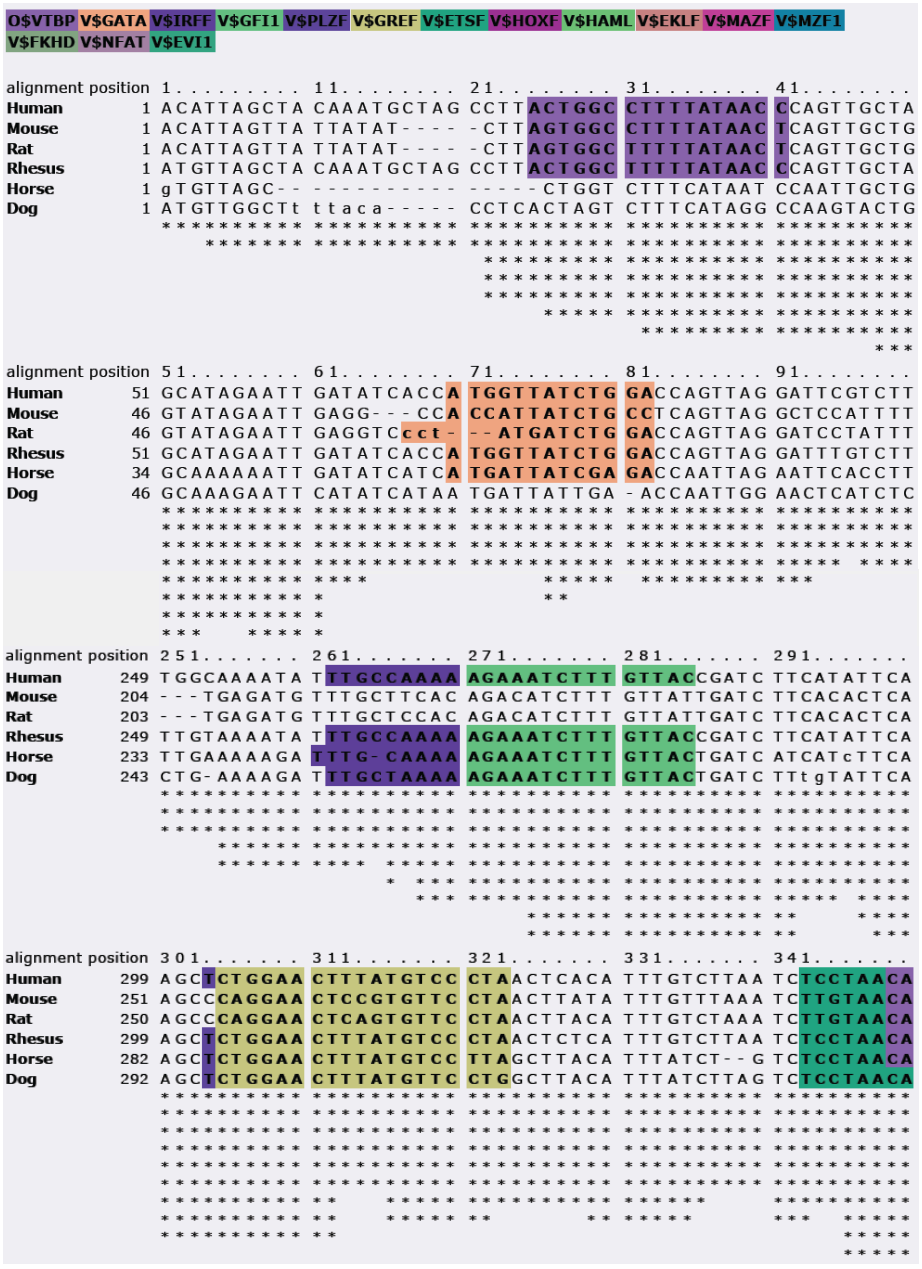
Supplementary Figure 2. (Cont.)



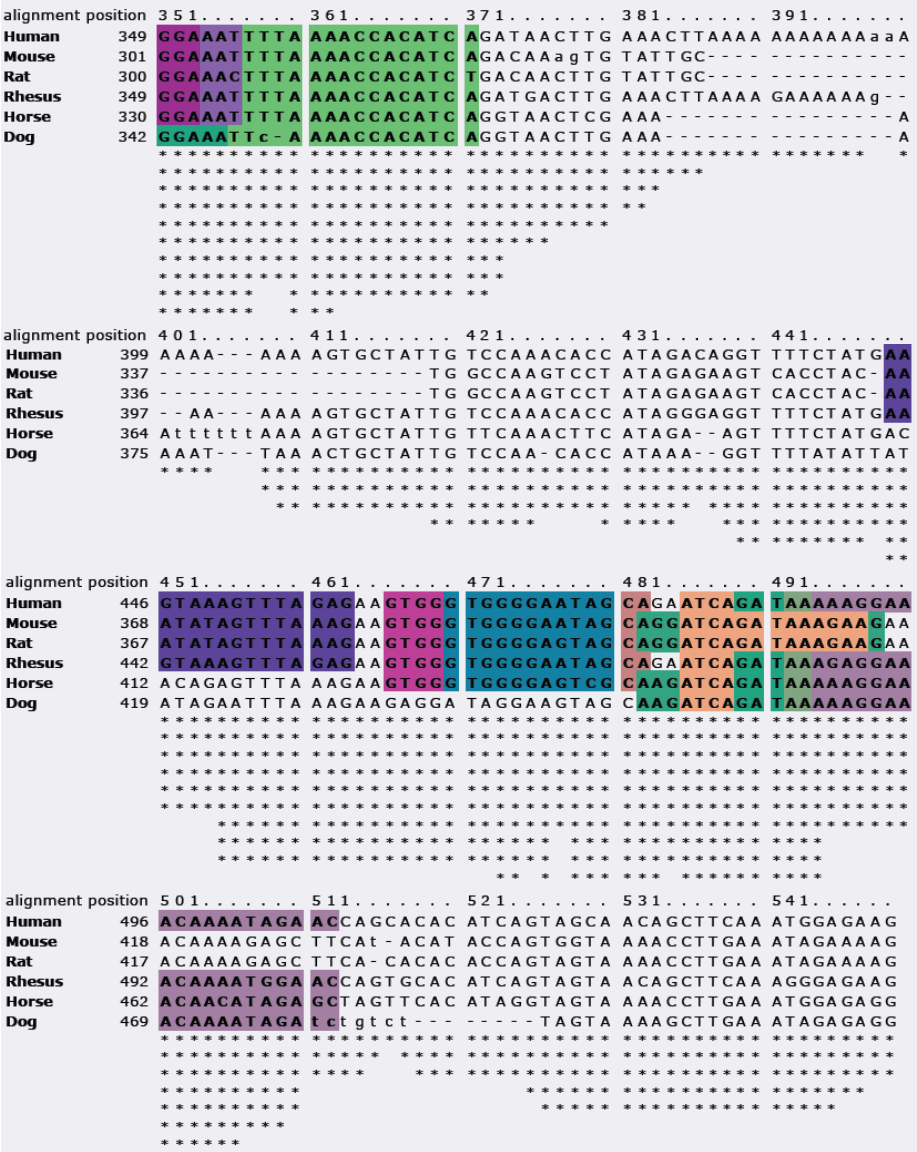
Supplementary Figure 3. Direct results of *Genomatix DiAlign Plus TF* for CNS3.

Aligned Sequences:

No.	Sequence Name	Sequence Description	Length
1	Human	Human Mar. 2006 chr12:9817153-9817696 (+)	544 bp
2	Mouse	Mouse Jul. 2007 chr6:129254105-129254569 (+)	465 bp
3	Rat	Rat Nov. 2004 chr4:166551105-166551568 (+)	464 bp
4	Rhesus	Rhesus Jan. 2006 chr11:9986085-9986624 (+)	540 bp
5	Horse	Horse Jan. 2007 chr6:37026523-37027032 (+)	510 bp
6	Dog	Dog May 2005 chr27:39198128-39198636 (-)	509 bp



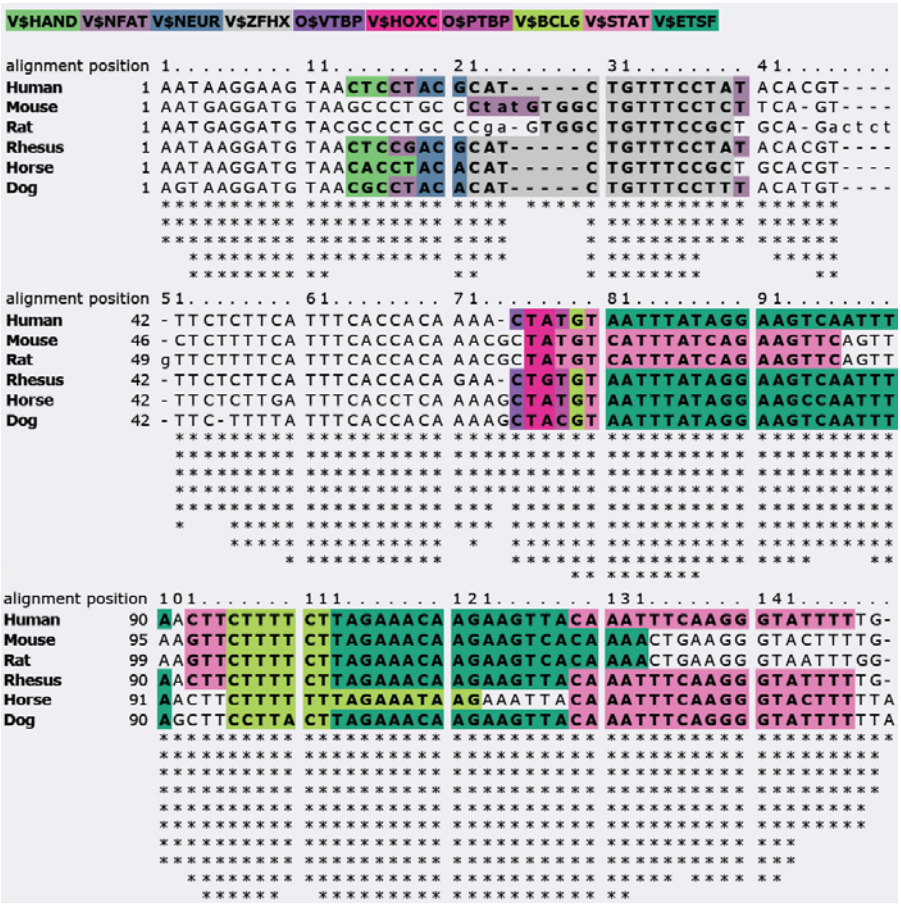
Supplementary Figure 3. (Cont.)



Supplementary Figure 4. Direct results of *Genomatix DiAlign Plus TF* for CNS4.

Aligned Sequences:

No.	Sequence Name	Sequence Description	Length
1	Human	Human Mar. 2006 chr12:9837823-9837990 (+)	168 bp
2	Mouse	Mouse Jul. 2007 chr6:129265841-129266019 (+)	179 bp
3	Rat	Rat Nov. 2004 chr4:166559923-166560104 (+)	182 bp
4	Rhesus	Rhesus Jan. 2006 chr11:10005693-10005860 (+)	168 bp
5	Horse	Horse Jan. 2007 chr6:37034347-37034516 (+)	170 bp
6	Dog	Dog May 2005 chr27:39192060-39192228 (-)	169 bp



CD69 Gene Is Differentially Regulated in T and B Cells by Evolutionarily Conserved Promoter-Distal Elements[1]

Berta N. Vazquez,*† Teresa Laguna,† Juan Carabana,‡ Michael S. Krangel,‡ and Pilar Lauzurica[2]*†

CD69 is a type II C-type lectin involved in lymphocyte migration and cytokine secretion. CD69 expression represents one of the earliest available indicators of leukocyte activation and its rapid induction occurs through transcriptional activation. In this study we examined the molecular mechanism underlying mouse CD69 gene transcription in vivo in T and B cells. Analysis of the 45-kb region upstream of the CD69 gene revealed evolutionary conservation at the promoter and at four noncoding sequences (CNS) that were called CNS1, CNS2, CNS3, and CNS4. These regions were found to be hypersensitive sites in DNase I digestion experiments, and chromatin immunoprecipitation assays showed specific epigenetic modifications. CNS2 and CNS4 displayed constitutive and inducible enhancer activity in transient transfection assays in T cells. Using a transgenic approach to test CNS function, we found that the CD69 promoter conferred developmentally regulated expression during positive selection of thymocytes but could not support regulated expression in mature lymphocytes. Inclusion of CNS1 and CNS2 caused suppression of CD69 expression, whereas further addition of CNS3 and CNS4 supported developmental-stage and lineage-specific regulation in T cells but not in B cells. We concluded CNS1–4 are important *cis*-regulatory elements that interact both positively and negatively with the CD69 promoter and that differentially contribute to CD69 expression in T and B cells. *The Journal of Immunology,* **2009, 183: 0000–0000.**

Leukocyte activation involves coordinated changes in the expression of key genes involved in the inflammatory cascade and leukocyte migration to promote effective immune responses against diverse pathogens and malignant cells. Recently, several reports using in vivo animal models have highlighted the role of the CD69 membrane molecule in both cytokine gene regulation and cell migration upon leukocyte activation. CD69 has been shown to be involved in the inhibition of lymphocyte egress from lymphoid organs in response to IFN-$\alpha\beta$ through a mechanism that involves down-regulation of S1P1 receptors (1). Overexpression of CD69 in transgenic (Tg)[3] mice supports a role in thymocyte migration (2, 3). CD69-deficient mice display enhanced resistance to MHC class I^- tumor growth and increased susceptibility to collagen induced arthritis and *Listeria monocytogenes* infection, associated with increased cellular recruitment and altered cytokine production and apoptosis (Refs. 4, 5; J. Vega-Ramos, E. Alari-Pahissa, J. Valle, E. Carrasco-Marín, E. Esplugues, M. Borrás, C. Martinez-A, and P. Lauzurica, manuscript submitted for publication). Moreover, in vivo blocking of CD69 with mAbs resulted in exacerbated autoimmune and antitumor responses (6). Recently, it has been demonstrated that $CD69^+CD4^+CD25^-$ cells represent a new subset of regulatory T cells involved in tumor-induced immunosuppression (7). Although CD69 is expressed during lymphocyte development (8), positive and negative selection of thymocytes is normal in CD69 deficient mice and only minor alterations in the pre-B cell compartment have been detected (9).

The CD69 gene is located in the NK complex on mouse chromosome 6 and human chromosome 12. This complex includes a variety of genes encoding C-type lectins with diverse expression patterns and functions in the immune system (10). CD69 is expressed on the surface of activated leukocytes through a mechanism that involves ras and raf activation and calcium release (11, 12). A variety of agents, including anti-CD3 Abs, TNF-α, IFN-$\alpha\beta$, poly(I:C), or phorbol esters can up-regulate CD69 in vitro. Transcripts are detected as early as 30 min after T cell stimulation and cell surface protein is observed 3 h later (13). CD69 transcription, however, is transient and returns to an "off" state at later times (14). Transient transfection experiments showed that both the mouse and human CD69 promoters can direct reporter transcription in cells stimulated with PMA plus ionomycin (Io) (14, 15). C*is* elements contributing to this inducibility were mapped to the proximal promoter region and these elements were shown to interact with transcription factors Erg-1, Erg-3, ATF-3/CREB, and AP-1 upon stimulation (16). Basal CD69 transcription was also detected by transient transfection of mouse and human promoter constructs and was attributed to the -78 to $+16$ region of the human CD69 gene. Interestingly, the transcription factor Sp1 was shown to constitutively bind to this region. In another study, an NF-κB motif at position -223 of the human CD69 promoter was shown to be required for transcriptional induction of CD69 in response to TNF-α (17).

Transient transfection assays do not account for potential influences of the chromatin environment, which may require the action

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[3] Abbreviations used in this paper: Tg, transgenic; Io, ionomycin; HA, hemagglutinin; CNS, conserved noncoding sequence; ChIP, chromatin immunoprecipitation; CAD, carbamoyl-transferase dihydroorotase; HS, hypersensitive site; DP, double positive; SP, single positive; MFI, mean fluorescence intensity.

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of distal enhancers, silencers, and insulators for efficient gene expression, and may require DNA methylation and histone modifications to regulate access to transcription factors. Epigenetic regulation has been shown to be critical for inducible expression of a variety of immune system genes, including IL-4, IFN-γ, IFN-β, and IL-12 (18–21). Therefore, the goal of this study was to define the epigenetic changes and *cis*-acting elements important for regulated CD69 gene expression in lymphocytes in vivo. We searched for distal regulatory elements using cross-species sequence comparison, DNase I hypersensitivity mapping, and transcriptional activity analysis in transient transfection assays and we then used a Tg approach to test the functional significance of candidate *cis*-regulatory elements in vivo. Our results indicate that unusual as well as common transcriptional regulatory mechanisms control expression of the CD69 gene in different lymphocyte populations.

Materials and Methods

Comparative genomic analysis

Sequence comparison of mouse, human, and dog CD69 was performed using VISTA Browser from the Lawrence Berkeley National Laboratory (available at http://pipeline.lbl.gov/cgi-bin/gateway2).

DNase I hypersensitivity assay

Rag2$^{-/-}$ × *Tcrb* Tg mice (Rxβ) and hemagglutinin (HA)-TCR Tg mice were described previously (22, 23). RBC were lysed in 0.15 M NH_4Cl, 1 mM $KHCO_3$, 1 mM Na_2-EDTA (pH 7.4) for 3 min at 4°C and subsequently washed in 30 ml of cold PBS. Thymocytes (10^7/ml) were permeabilized with 0.067 mg/ml lysolecithicin in buffer C (0.15 M sucrose, 80 mM KCl, 30 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 5 mM $CaCl_2$) for 5 min. DNase I was added at a final concentration of 0, 4, 8, 12, 16, 20, 24, 28, or 32 U/ml for 10 min on ice. Reactions were stopped by the addition of EDTA, SDS, and proteinase K to final concentrations of 10 mM, 0.4% (w/v), and 0.4 mg/ml, respectively, and were incubated overnight at 37°C. DNA was purified by phenol, phenol/chloroform, and chloroform extractions and ethanol precipitation, taking care not to shear the genomic DNA. Purified DNA (10 μg) was incubated overnight at 37°C with an excess of *Eco*RI restriction enzyme. Digests were separated by 0.7% (w/v) agarose gel electrophoresis and were analyzed by Southern blot with ^{32}P-labeled DNA probes (primers to generate probes; supplemental Table I).[4]

Reporter constructs and Tg mice

hCD2 reporter constructs were generated by modification of the mCD8 reporter vector (24). To generate construct 1 (see Fig. 4*B*), the promoter of mCD69 (−645/+1) was PCR amplified (Expand High Fidelity PCR System; Roche) and cloned into pstBlue vector (Vector Acceptor Kit; Novagen). Restriction sites introduced during PCR amplification were used to excise the mCD69 promoter from this plasmid with *Apa*I and *Cla*I and ligate it to the *Apa*I/*Cla*I-digested hCD2 construct. To ensure that we incorporated conserved sequences and DNase I hypersensitive sites, primers for genomic regions encompassing conserved noncoding sequence (CNS)1 (233 bp), CNS2 (451 bp), CNS3 (439 bp), and CNS4 (181 bp) were designed (primer sequences, supplemental Table I). PCR products for CNS1–4 fragments were 807, 932, 600, and 707 bp in length, respectively. To generate construct 2, CNS1 and CNS2 were first combined into pstBlue cloning vector. CNS2/CNS1 fragment was then excised from this construct using *Apa*I and *Xba*I and inserted into *Xba*I/*Apa*I sites upstream of the mCD69 promoter in construct 1. Similarly, to make construct 3, CNS3 and CNS4 were combined into pstBlue, digested with *Xba*I, and inserted into the *Xba*I site upstream of CNS2 in construct 2. Importantly, *Not*I restriction sites were flanking all three hCD2 reporter constructs. Construct 1 had a size of 3.7 kb and was separated from vector backbone using *Apa*I/*Not*I digestion. Construct 2 was 5.4 kb and was separated with *Xba*I/*Not*I digestion and construct 3, with a size of 6.7 kb, was separated with *Not*I digestion. Purified DNA was then microinjected into fertilized eggs. Founders were identified by PCR and copy number was determined by quantitative real-time PCR. Input DNA was determined using primers ExonIIIF and ExonIIIR (primers sequences, supplemental Table I). Handling of mice and experimental procedures were in accordance with institutional requirements for animal care and use.

[4] The online version of this article contains supplemental material.

Purification of cell populations and cell culture

To enrich for T cell populations, lymph node cell suspensions were passed through a nylon wool column (Polysciences) following the manufacturer's instructions. T cell purity, determined by flow cytometry, was >85%. Splenocytes of TCR$\beta^{-/-}$TCR$\delta^{-/-}$ mice were used to obtain B cells (>95% B220$^+$ by flow cytometry).

Before chromatin immunoprecipitation (ChIP) analysis of peripheral lymphocyte populations, cells were stimulated in vitro with 10 ng/ml PMA (Sigma-Aldrich) and 0.5 μM calcium ionophore (Sigma-Aldrich) for 6 h at 37°C. CD69 expression was then analyzed by flow cytometry.

For hCD2 analysis in peripheral lymphocytes, splenocytes of wild-type and Tg mice were activated by incubation with 10 ng/ml PMA and 1 μM Io or 5 μg/ml plate-bound anti-CD3 (clone 145-2C11; eBioscience) and anti-CD28 (clone 37.51; eBioscience) mAbs overnight at 37°C. In experiments using PMA plus Io, data were collected from experiments in which activated lymphocytes were >90% CD69$^+$.

Poly(I:C) treatment

Mice were injected i.p. with 500 μg of poly(I:C) (Sigma-Aldrich). After 18 h of treatment, spleens were obtained from animals and lymphocyte suspensions were prepared for FACS analysis.

Chromatin immunoprecipitation

For analysis of thymocytes, immunoprecipitations were performed on purified mononucleosomes as described previously (25). Briefly, thymocytes were lysed and nuclei were treated with micrococcal nuclease to produce a partial chromatin digest. After removal of linker histone H1, chromatin was fractionated on a sucrose gradient. For analysis of peripheral lymphocytes, immunoprecipitations were performed on paraformaldehyde-cross-linked chromatin prepared as described previously (26). Sonication was used to obtain DNA fragments ranging from 300 to 500 bp. With either approach, anti-diacetylated H3, anti-dimethylated H3 K4, and control rabbit-IgG Abs (Upstate Biotechnology) were used for immunoprecipitation and bound and input fractions were quantified using SYBR Green real-time PCR (Roche). Analysis of the constitutively active carbamoyl-transferase dihydroorotase (CAD) gene was used to normalize values of different samples. Primer sequences are provided in supplemental Table I.

Flow cytometry

Cell suspensions from spleen, thymus, and bone marrow were stained with FITC-CD69 (BD Biosciences), PE-hCD2 (Caltag Laboratories), PerCP-CD4 (BD Biosciences), allophycocyanin-CD8 (BD Biosciences), FITC-CD24 (HSA; eBioscience), PE-Cy5 IgM (eBioscience), or allophycocyanin-B220 (eBioscience) Abs. Data were collected on a FACSCalibur or FACSCanto (BD Biosciences) and were analyzed using FlowJo software.

Luciferase reporter constructs and cell transfections

For the firefly luciferase vector we used the pXPG vector (27). Primers used to amplify fragments were the same as for the hCD2 reporter constructs with modifications in the restriction sites (primer PromF contained a *Xho*I restriction site and other primers a *Bam*HI restriction site). The CD69 promoter was digested with *Xho*I and *Bam*HI and ligated to *Xho*I/*Bam*HI-digested pXPG-Luc vector. CNS1–4 fragments were digested with *Bam*HI and ligated to *Bam*HI-digested CD69 prom-pXPG-Luc vector. Construct sequences were confirmed by restriction enzyme digestion and sequence analysis.

For transient transfection assays, a total of 5×10^5 Jurkat cells were plated into a 24-well plate and transfected with 1 μg of specific firefly luciferase test plasmid, 20 ng of pRL-TK *Renilla* luciferase control plasmid, and 4 μl of SuperFect (Qiagen) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were stimulated with 10 ng/ml PMA and 1 μM Io or 5 μg/ml plate-bound anti-CD3 (clone OKT3; eBioscience) and anti-CD28 (clone CD28.2; eBioscience) mAbs or were mock incubated. A luciferase assay was performed 24 h later using the Dual-Luciferase Reporter Assay System (Promega). Transfections were performed in duplicate and values were normalized to *Renilla* luciferase activity.

Results

Conservation of the CD69 locus

CD69 is a type II transmembrane C-type lectin encoded in the NK complex on mouse chr6 and human chr12 (Fig. 1*A*). The gene spans ~7.5 kb and contains 5 exons. The first two exons encode the cytoplasmic and transmembrane domains, and exons III, IV,

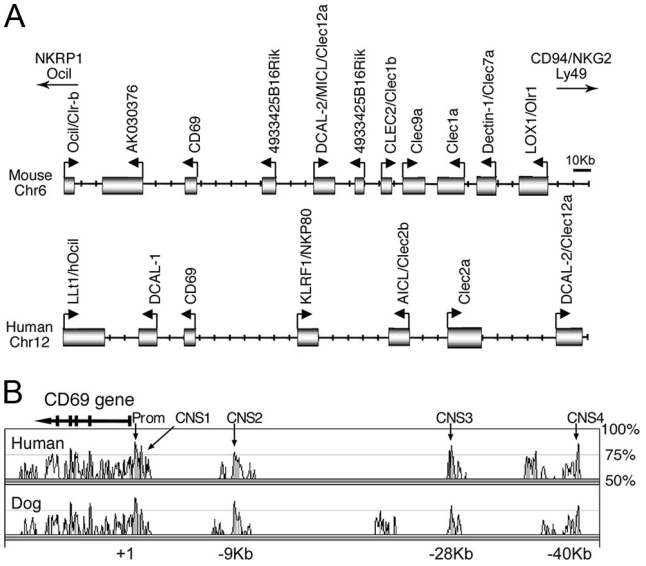

FIGURE 1. Genomic organization and conservation of the CD69 locus. *A*, Genomic organization of the human and mouse CD69 gene and neighboring genes in the NK complex. The diagram is drawn to scale according to the most recent gene annotations (July 2007 for mice and March 2006 for human) at the University of California Santa Cruz website (http://genome.ucsc.edu). Boxes are genes and arrows indicate transcriptional initiation sites and orientation. *B*, VISTA Browser diagram identifying conserved noncoding sequences upstream of the CD69 gene. The mouse sequence is shown on the *x*-axis and percentage of similarity to the human and dog genomes is shown on the *y*-axis. Noncoding sequences of at least 100 bp long with >70% sequence identity are indicated with arrows.

and V encode the extracellular portions of the molecule. The defined murine CD69 promoter (−656 to +1 relative to the transcription start site) is the only *cis*-acting element known to regulate CD69 expression (13). We wanted to identify other potential *cis*-acting sequences involved in CD69 gene regulation. Because cross-species genome analysis has been useful for this purpose (28), we compared mouse, human, and dog genomic sequences by the means of VISTA Browser (29). Using the default parameters for defining a conserved CNS element (70% identity over 100-bp length), four elements upstream of the CD69 gene were identified (Fig. 1*B*). CNS1 was upstream of and contiguous to the promoter, and CNS2, CNS3, and CNS4 were 9, 28, and 40 kb away from the main site of transcription initiation, respectively. Marked conservation was also observed at the promoter. Because there are substantial differences in genomic organization of the human and mouse NK complexes upstream of CD69 (Fig. 1*A*), we hypothesized that CNS1–4 may regulate CD69 rather than neighboring gene expression.

Accessibility of the CD69 locus

Chromatin regulatory regions are typically characterized by DNase I hypersensitive sites (HSs) that reflect NF binding and disrupted nucleosome organization. We conducted DNase I HS assays and Southern blots to determine whether CNS1–4 represented DNase HSs. CD69 is up-regulated by thymocyte-positive selection, and different TCR Tg mouse strains express characteristic quantities of CD69 based on both the selecting background and the strength of TCR-MHC/peptide interactions (30). We used thymocytes from B10.D2 H-2K^{d} mice expressing a Tg TCR specific for a HA peptide presented by H-2K^{d} to isolate thymocytes with high CD69 expression and thymocytes from Rag2$^{-/-}$ mice that express a *Tcrb* transgene (Rxβ) to isolate thymocytes with low CD69 expression

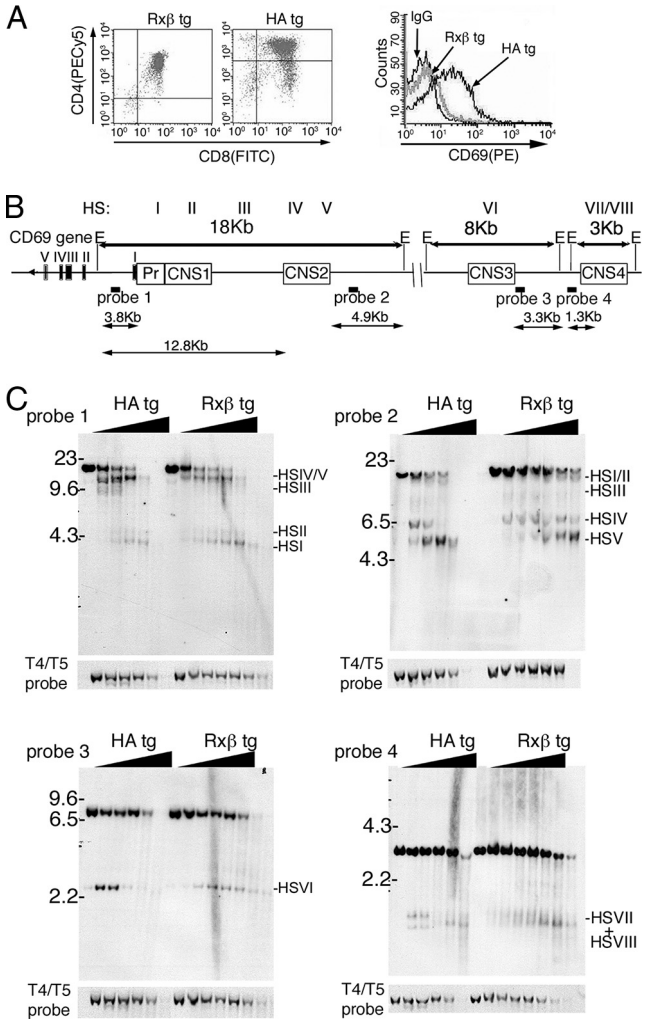

FIGURE 2. Conserved noncoding sequences are constitutive HSs. *A*, Flow cytometry analysis showing CD69 expression in Rxβ and HA Tg thymi. *B*, Map of the CD69 locus showing locations of CNSs and the genomic fragments expected from *Eco*RI digestion. Probes used for Southern blot are shown and HSs are indicated. *C*, Representative DNase I analyses using the indicated probes. Total thymocytes from Rxβ and HA thymocytes were treated with increasing amounts of DNase I. *Eco*RI digested DNA was examined by Southern blot. Size markers and relevant bands are denoted.

(Fig. 2*A*). Total thymocytes from these strains were treated with varying concentrations of DNase I, and genomic DNA was extracted, digested with *Eco*RI, and analyzed by Southern blot (Fig. 2*B*). A site within the inactive trypsinogen gene was used as an internal control to compare chromatin digestion in different samples. Probes 1 and 2, which hybridized to different ends of the same genomic *Eco*RI fragment (Fig. 2*B*), detected five distinct HSs (Fig. 2*C*, *upper panels*). HSI and HSII corresponded to the CD69 promoter and CNS1, respectively, whereas HSIV and HSV mapped to CNS2. HSIII was evident as a cluster of weak HSs that was only detected in Rxβ and that mapped to a less conserved sequence between CNS1 and CNS2. HSVI, corresponding to CNS3, was detected using probe 3 (Fig. 2*C*, *lower left panel*). Probe 4 revealed weak HSVII and HSVIII that corresponded to CNS4 (Fig. 2*C*, *lower right panel*). Hence, these experiments identified eight distinct DNase I HSs mapping predominantly to the defined CNSs. CD69^{+} and CD69^{-} cells presented similar patterns of DNase I sensitivity.

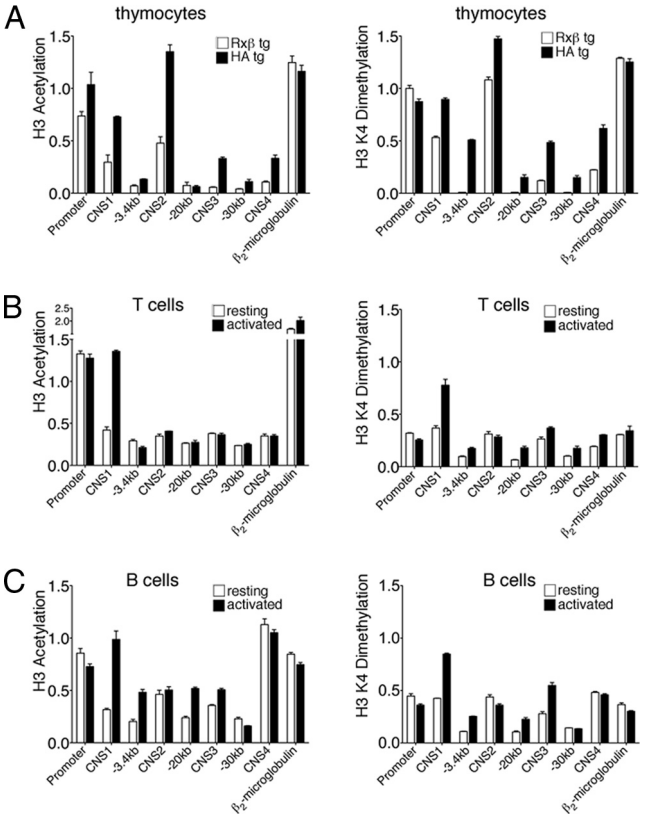

FIGURE 3. Epigenetic profile of the CD69 locus in T and B cells. ChIP with antisera against acetylated H3 and dimethylated H3 K4. *A*, ChIP performed on purified mononucleosomes obtained from total thymocytes of Rxβ and HA Tg mice. *B*, ChIP performed on sonicated chromatin from purified T cells either unstimulated or stimulated with 10 ng/ml PMA plus 0.5 μM Io for 6 h. Purity as determined by flow cytometry was >85%. *C*, ChIP performed on sonicated chromatin from B cells isolated from TCR$\delta^{-/-}\beta^{-/-}$ mice, either unstimulated or stimulated with 10 ng/ml PMA plus 0.5 μM Io for 6 h. Flow cytometry analysis showed that >95% of spleen cells were B220$^+$. Bars represents the abundance of indicated DNA sequences in immunoprecipitated samples and are expressed as the ratio of immunoprecipitated DNA and input DNA normalized to the abundance of the constitutively active control CAD gene. The data are representative of two experiments and are expressed as the means ± SEM of triplicate PCRs.

CD69 locus histone modifications

One mechanism by which chromatin structure participates in the regulation of gene expression is through the modification of histone tails (31). Histone H3 acetylation and lysine 4 dimethylation are associated with active and poised chromatin, respectively (32). We assessed their levels by ChIP at conserved genomic regions (CD69 proximal promoter, CNS1, CNS2, CNS3, and CNS4) as well as at several nonconserved regions (−3.4, −20, and −30 kb). The β_2-microglobulin gene was used as positive control.

Mononucleosomes were prepared from Rxβ and HA Tg thymocytes and were then immunoprecipitated with anti-acetylated histone H3, anti-dimethylated histone H3 K4, or control IgG Abs. Coprecipitated DNA was purified and subjected to real-time PCR to quantify the recovery of regions of interest (Fig. 3*A*). Results are expressed as the ratio of immunoprecipitated and input DNA normalized to the constitutively active control gene CAD. The promoter demonstrated high levels of H3 acetylation and H3 K4 dimethylation in both CD69$^-$ Rxβ thymocytes and CD69$^+$ HA Tg thymocytes. CD69$^-$ Rxβ thymocytes demonstrated moderate acetylation at CNS1 and CNS2, and very low levels at CNS3 and CNS4. CD69$^{+/+}$ HA Tg thymocytes displayed increases in H3 acetylation at all of these sites. A similar overall pattern was observed for H3 K4 dimethylation, with the exception that this modification was only modestly increased at CNS2 in CD69$^+$ cells. With one exception (H3 K4 dimethylation at position −3.4 kb in CD69$^+$ thymocytes), the two modifications were very low at all nonconserved sites tested.

To investigate CD69 locus chromatin structure in peripheral lymphocytes, lymph node T cells and splenic B cells were treated in vitro with PMA and Io for 6 h to up-regulated CD69 expression or were left untreated. Chromatin was then cross-linked with paraformaldehyde, fragmented by sonication, and immunoprecipitated as above. As in thymocytes, acetylation of H3 and dimethylation of H3 K4 were enriched at the promoter in resting and activated T and B cells (Fig. 3, *B* and *C*). Also as in thymocytes, H3 acetylation and H3 K4 dimethylation increased substantially at CNS1 upon activation of both peripheral T and B cells (Fig. 3, *B* and *C*). However, unlike in thymocytes, we detected only very low H3 acetylation at CNS2, CNS3, and CNS4 in both resting and activated peripheral T cells. Moreover, H3 K4 dimethylation at these sites, although mildly elevated in resting T cells, was not inducible. B cells displayed increased amounts of both modifications at CNS2, CNS3, and CNS4, but only CNS3 showed inducible modification. Moreover, in striking contrast to thymocytes, H3 acetylation and H3 K4 dimethylation were constitutively high at CNS4 in B cells.

The above experiments revealed three important aspects of CD69 gene chromatin structure. First, the CD69 promoter is constitutively associated with active chromatin modifications in all three cell types. Second, CNS1 becomes hyperacetylated and hypermethylated upon CD69 induction in all three cell types. Third, histone modifications at CNS2, CNS3, and CNS4 undergo dynamic changes during T cell development but are differentially modified in peripheral T and B cells. This suggests the possibility of distinct mechanisms of CD69 gene regulation in the various cell types.

Analysis of promoter and CNS1–4 function

The correlation between DNA conservation and the presence of HSs and positive histone marks at the promoter and CNS1–4 prompted us to test their regulatory properties. We prepared luciferase reporter constructs under the control of the CD69 promoter linked to CNS fragments (Fig. 4*A*). Plasmids were transiently transfected into Jurkat cells, cultured for 24 h, and then stimulated or not with PMA/Io for 24 h more before cells extracts were harvested for luciferase activity. Stimulation conferred a 4-fold increase in the activity of the CD69 promoter. Inducible activity of the CD69 promoter, however, was greatly enhanced in the presence of CNS2. This fragment conferred a 2-fold increase in basal activity and 20-fold increase over basal activity under conditions of stimulation. CNS4 conferred an ~5-fold increase in basal transcription activity and a further 2-fold increase under conditions of stimulation. In contrast, reporter activity was not altered when CNS1 or CNS3 was linked to the CD69 promoter. Luciferase activity of these constructs was also determined upon stimulation with immobilized Abs against CD3 and CD28 (supplemental Fig. 1). CNS2 was found to confer inducibility to this stimulus, although the magnitude (3-fold) was lower than with PMA/Io. These results provide initial evidence that CNS2 and CNS4 may be enhancer elements for CD69 gene transcription.

To assess their in vivo functional properties, Tg mice were generated using a hCD2 expression construct as a reporter. Transgenic constructs containing the mCD69 promoter alone upstream of hCD2 (construct 1) or together with all four CNSs (construct 3)

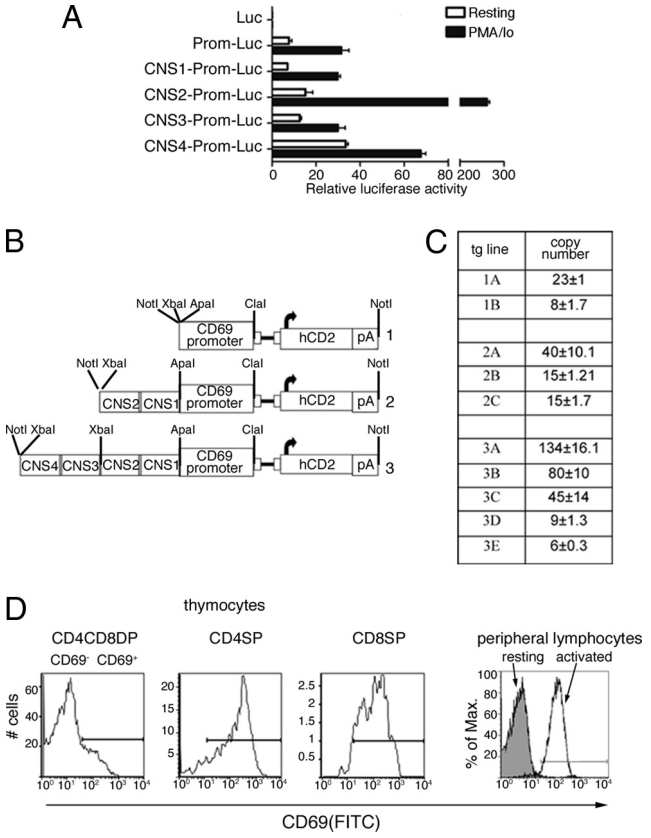

FIGURE 4. Characterization of CNS function in transfected cells and Tg mice. *A*, Luciferase activity of reporter constructs in unstimulated or stimulated Jurkat cells. Results are expressed as the mean ± SEM of duplicate transfections and are representative of three experiments. *B*, Representation of hCD2 reporter constructs used to generate Tg mice. *C*, Copy numbers of Tg lines were determined by real-time PCR. Values for construct 1 represent the average amplification using hCD2 primers, values for construct 2 represent the average amplification using CNS1 and hCD2 primers, and values for construct 3 represent the average amplification using CNS4, CNS3, CNS1, and hCD2 primers. *D*, Flow cytometric analysis of CD69 expression in thymus and resting and activated peripheral lymphocytes, indicating gating for $CD69^+$ and $CD69^-$ populations.

were generated (Fig. 4*B*). Due to the strong enhancer activity observed for CNS2 element in luciferase assays, Tg mice containing CNS1 and CNS2 upstream of the promoter (construct 3) were also generated. Two Tg lines were obtained for construct 1, three for construct 2, and five for construct 3 (Fig. 4*C*). Copy numbers were determined by quantitative real-time PCR (Fig. 4*C*).

We assessed the magnitude and fidelity of hCD2 reporter expression on gated $CD69^+$ and $CD69^-$ thymocytes and on purified resting or activated mature lymphocyte populations by flow cytometry (Fig. 4*D*). Tg lines 1A-23 and 1B-8 (containing 23 and 8 copies of the transgene, respectively) that contained only the CD69 promoter expressed hCD2 in both thymocytes and peripheral lymphocyte populations (Fig. 5*A* and supplemental Figs. 2*A* and 3*A*). hCD2 expression in double-positive (DP) and single-positive (SP) thymocyte populations was strictly correlated with endogenous CD69 expression, suggesting that the promoter was sufficient to confer specificity to CD69 expression during positive selection (Fig. 5*A*). However, aberrant expression of hCD2 was observed in resting peripheral T (Fig. 5*A*) and B (Fig. 6*A*) lymphocytes, suggesting the need for additional elements.

We tested for inducibility of the mCD69 promoter by overnight activation of isolated peripheral T and B lymphocytes with PMA and Io. Because even resting cells expressed hCD2, we used the mean fluorescence intensity (MFI) of the whole population to measure changes in hCD2 expression. Inducibility was calculated as the ratio of the MFI for the hCD2 staining between activated and resting cells. We detected 1.5- to 2.5-fold hCD2 inducibility in activated T cells (Fig. 5, *B* and *C*) and 1.5- to 2-fold inducibility in activated B cells (Fig. 6*B*), suggesting that promoter elements support some inducibility in vivo.

Unexpectedly, inclusion of CNS1 and CNS2 upstream of the CD69 promoter (Tg lines 2A-40, 2B-15, and 2C-15) suppressed hCD2 expression in both thymocytes and peripheral T and B cell populations (Figs. 5*A* and 6*A* and supplemental Figs. 2*B* and 3*B*). hCD2 expression was substantially reduced in $CD69^+$ DP and SP thymocytes, and it was essentially eliminated in resting and activated peripheral $CD4^+$ and $CD8^+$ T cells (Fig. 5*A*). Expression was also eliminated in resting and activated peripheral B cells in two of the three Tg lines (Fig. 6*A* and supplemental Fig. 3*B*). These data suggest that CD69 gene expression in lymphocytes is regulated by silencer elements mapping to CNS1 or CNS2.

In contrast to the results obtained with construct 2, Tg lines carrying construct 3, including the CD69 promoter and all four CNSs (Tg lines 3A-134, 3B-80, 3C-45, 3D-9 and 3E-6), revealed a recovery of hCD2 expression (Figs. 5*A* and 6*A* and supplemental Figs. 2*C* and 3*C*). Expression of the hCD2 reporter correlated well with CD69 expression in all thymocyte populations, ranging from 92 to 22% in DP thymocytes and 89 to 15% in CD4SP and CD8SP thymocytes (supplemental Fig. 2*C*). Within the SP compartments, hCD2 expression also correlated with mCD69 expression, since down-regulation of CD69 and HSA in more mature SP thymocytes was associated with down-regulation of reporter expression (supplemental Fig. 4). Notably, high-copy Tg lines 3C-45, 3B-80, and 3A-134, but not low-copy lines 3E-6 and 3D-9, displayed variegated hCD2 expression indicative of genomic position effects. In the low copy lines hCD2 expression faithfully mimicked mCD69 expression, and expression levels were greater than those observed with construct 1, containing the promoter alone (compare Tg lines 1B-8 and 3E-6 in Fig. 5*A*).

Analysis of construct 3 expression in mature $CD4^+$ and $CD8^+$ T cells stimulated with PMA/Io revealed hCD2 expression to be restricted to activated cells in four of five Tg lines (3E-6, 3C-45, 3B-80, and 3A-134) with percentages ranging from 99 to 10% (supplemental Fig. 2*C*). However, hCD2 expression was inducible in all five lines (range, 23-fold to 3.5-fold), and inducibility was substantially higher than for Tg lines containing construct 1 with the promoter alone. Tg line 3E-6, with the lowest copy number, was the most tightly regulated, with negligible hCD2 expression in resting $CD4^+$ and $CD8^+$ T cells, and abundant hCD2 expression in the vast majority of activated $CD4^+$ and $CD8^+$ T cells (Fig. 5*A*). Inducibility was 14-fold and 15-fold in $CD4^+$ and $CD8^+$ T cells, respectively (Fig. 5, *B* and *C*). As in thymocytes, expression was highly variegated in the three Tg lines with the highest copy numbers (3C-45, 3B-80, and 3A-134) (supplemental Fig. 2*C*).

Expression of construct 3 in peripheral T cells was also inducible following direct engagement of TCR with anti-CD3 plus anti-CD28 Abs (Fig. 5*D*). Moreover, the kinetics of hCD2 expression paralleled mCD69 expression, since both were detected 2 h after stimulation, were maximally detected at 12–24 h, and expression was reduced thereafter (supplemental Fig. 5, *A* and *B*). Since CD69 expression can be induced in vivo by infectious agents that cause the production of type I IFNs, we also analyzed hCD2 induction following in vivo administration of poly(I:C) (supplemental Fig. 6). The hCD2 expression was induced after poly(I:C) treatment, indicating that CNSs can also respond to stimuli distinct from the TCR pathway. These results

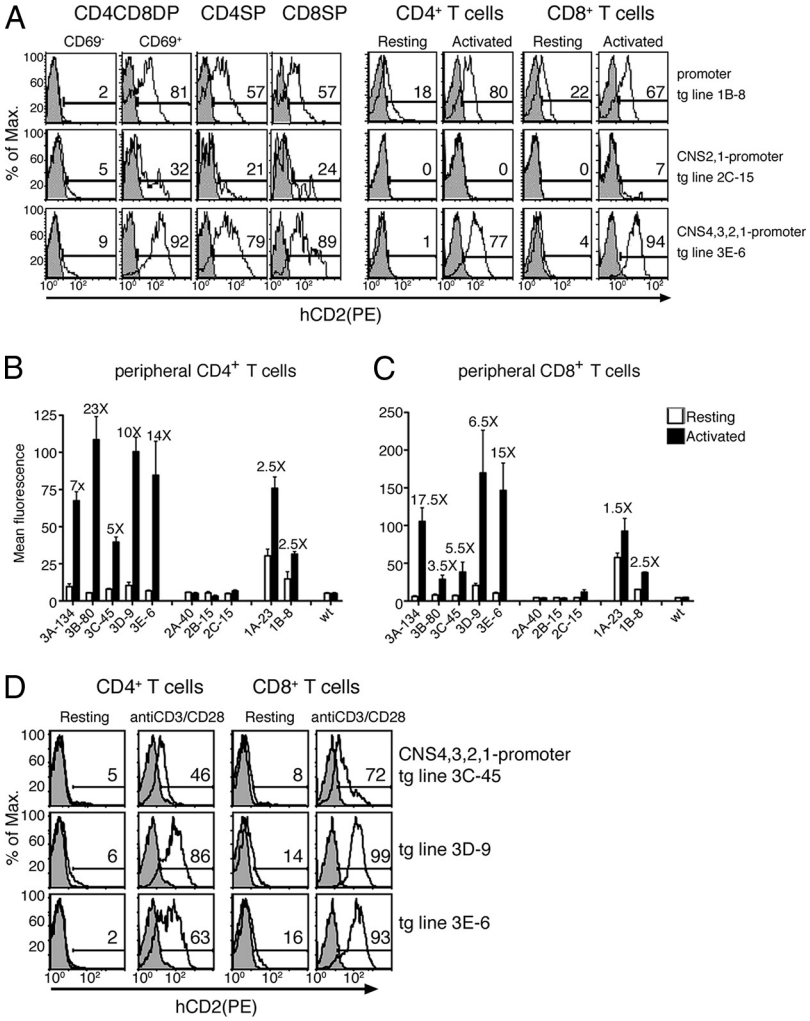

FIGURE 5. Reporter expression in thymocytes and peripheral T cells of Tg mice. *A*, Cytometric analysis of hCD2 expression in Tg lines 1B-8, 2C-15, and 3E-6 from thymocytes ($CD69^+$ and $CD69^-$ CD4CD8DP, CD4SP, and CD8SP) and splenocytes (resting or activated with PMA/Io, $CD4^+$, and $CD8^+$) (open histograms). Analyses of control non-Tg mice are also shown (filled histograms). *B* and *C*, MFI of the hCD2 expression in resting and activated peripheral $CD4^+$ T cells and $CD8^+$ T cells. Values reflect the means ± SEM of 5–10 mice. For each Tg line, fold-increase in MFI in response to activation is indicated above the bars. *D*, Cytometric analysis of hCD2 expresion in Tg lines 3C-45, 3D-9, and 3E-6 activated or not with plate-bound anti-CD3/CD28 Abs. Analyses of control non-Tg mice are also shown (filled histograms).

suggest that the combination of CNS1–4 contributes to regulated CD69 expression in both thymocytes and peripheral T cells under a variety of activation conditions.

We further examined the expression of hCD2 in B cells. The combination of CNS1–4 clearly enhanced hCD2 expression as compared with the promoter alone (Fig. 6*A* and supplemental Fig. 3*C*). However, hCD2 expression was not concordant with mCD69 expression, since substantial numbers of resting B cells were $hCD2^+$ in all five Tg lines (range, 22–79%) (supplemental Fig. 2*C*). This may reflect the constitutive and abundant H3 acetylation and H3 K4 dimethylation detected at CNS4 in B cells (Fig. 3*C*). Stimulation with PMA and Io resulted in 2- to 2.5-fold inducibility in the various lines (Fig. 6*B*). However, the failure to appropriately suppress expression in resting B cells suggests that there are different requirements for regulated CD69 gene expression in B and T cells.

Because dysregulated expression of hCD2 was observed in mature B cells, we evaluated whether CNSs and the promoter may have an effect on hCD2 expression at different stages of B cell development (supplemental Fig. 7). Results indicate that hCD2 expression was already present in $B220^{int}IgM^+$ immature B cells in Tg lines containing the promoter alone or in combination with CNS1–4 elements and suggest that specific signals received at this stage of development induce the dysregulated expression of hCD2 that is also observed in mature B cells.

Collectively, these experiments indicate that the CD69 promoter has strong activity and by itself can faithfully direct CD69 transcription in developing thymocytes. CNS1 and CNS2 appear to repress promoter activity, whereas CNS3 and CNS4 appear to counteract this repression, resulting in more tightly regulated expression in peripheral T lymphocytes and enhanced expression in both thymocytes and peripheral T lymphocytes. The same elements are insufficient to faithfully direct CD69 expression in the B cell lineage.

Discussion

In this study we have provided insights into the mechanism that regulate CD69 gene expression in vivo by the identification of new *cis*-regulatory elements and analysis of their chromatin structure and function. We identified four conserved noncoding sequences upstream of the CD69 promoter. Chromatin and functional analyses indicated that the CD69 promoter adopts an open chromatin conformation and can direct reporter gene transcription in Tg mice. However, the reporter, unlike mCD69, was expressed in unstimulated peripheral lymphocytes, suggesting that other elements must participate to achieve appropriate specificity. We detected several DNase I hypersensitive sites that mapped to CNSs and that displayed epigenetic profiles that were distinct in T and B cells and dynamic during T cell development. Analysis of CNS function in transient transfection assays revealed constitutive and inducible enhancer activity for both CNS2 and CNS4, but no apparent activity for CNS1 and CNS3. However, we found that Tg mice bearing CNS1 and CNS2 reduced reporter expression in T and B cells and that this inhibition was only overcome by inclusion of CNS3

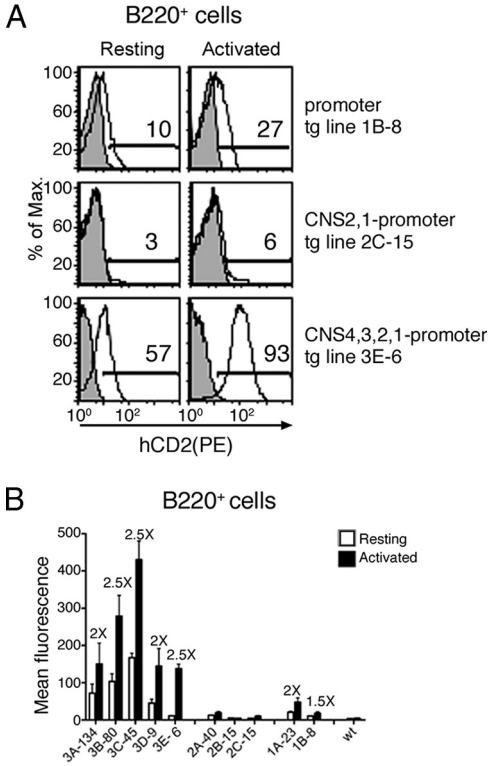

FIGURE 6. Reporter expression in resting and activated peripheral B cells of Tg mice. *A*, Cytometric analysis of hCD2 expression in Tg lines 1B-8 and 2C-15 from resting and activated states with PMA/Io B220$^+$ splenocytes (open histograms). Analyses of control non-Tg mice are also shown (filled histograms). *B*, MFI of the hCD2 expression in resting and activated peripheral B220$^+$ cells. Values reflect the meanS $\pm$ SEM of 5–10 mice. For each Tg line, fold increase in MFI in response to activation is indicated above the bars.

and CNS4. The combination of all four CNSs allowed for high level reporter expression that was appropriately regulated in T cells but not in B cells.

DNA sequence comparison has become a useful tool to identify specific remote elements that participate in the regulation of gene expression (19, 33, 34). We observed discrete islands of conservation in a 45-kb region upstream of the CD69 gene and found them to correspond to sites displaying hypersensitivity to DNase I digestion. It is noteworthy that these HSs were equally sensitive to DNase I digestion in CD69$^-$ and CD69$^+$ lymphocytes. CD69 is one of the earliest Ags expressed upon activation and, therefore, it is possible that these regions are occupied by proteins even before activation. In agreement with our results, the first genome-wide map of DNase I HSs in human CD4$^+$ T cells identified HSs lying within the CD69 promoter, CNS2, CNS3, and CNS4 in the human CD69 locus (35).

Previous work showed that the CD69 promoter can support rapid induction of reporter gene expression in transient transfection assays. Here we found that the CD69 promoter is always associated with an "active" chromatin configuration, even in situations where CD69 is not expressed. The promoter displayed high-level histone H3 acetylation and H3 K4 dimethylation in all thymocyte and peripheral lymphocyte populations. These two modifications have been shown to mark transcriptional competence of the IL-4 and IL-2 loci, respectively, in T cells (36, 37) and may be important to maintain transcriptional competence of the CD69 gene as well. Interestingly, genome-wide analysis of chromatin modifications in unstimulated human CD4$^+$ T cells demonstrated enrichment of both histone H3 K4 trimethylation and RNA polymerase II at the promoter, exon I, and intron I of mCD69 (34). These data suggest that, as for other genes that require rapid induction, the CD69 promoter may constitutively harbor a promoter-paused RNA polymerase II (38, 39). CNS1 is the distal region of the C69 promoter, and one notable result is that this region showed a peak of permissive histone marks that correlated with CD69 expression in all cell populations analyzed. However, the promoter alone showed inducible activity in both transient and Tg reporters, and at least in the case of transient assays, CNS1 had no influence on basal or inducible promoter activity. This suggests that inducible histone modifications at this site may depend on other elements.

Tissue- and developmental stage-specific gene expression depends on both activators and silencers. A well-studied case is that of CD4, in which a silencer represses the CD4 promoter and enhancer in double-negative and CD8 SP thymocytes (40, 41). Similarly, regulated IL-4 transcription requires the action of a 3′ silencer element that can suppress reporter gene expression in Tg mice (18) and whose germline deletion results in the aberrant expression of IL-4 in Th1 cells (34). Moreover, there are examples of bifunctional elements, which function as both enhancers and silencers in a developmental stage-specific way (42, 43). Our results from Tg mice indicate that CNS1, CNS2, or the combination of the two elements can play a repressive role in CD69 gene expression in thymocytes, as well as stimulated T and B cells. Nevertheless, CNS2 displayed strong, inducible enhancer activity when tested in isolation by transient transfection into Jurkat cells. Notably, the detected enhancer activity is consistent with the inducible histone H3 acetylation detected at this site in thymocytes. We suggest that CNS2 is an inducible thymocyte enhancer whose activity in the context of chromatin requires the activity of other CD69 regulatory elements. Remarkably, inclusion of CNS3 and CNS4 in Tg reporter constructs led to both a recovery of reporter gene expression and tightly regulated reporter gene expression in thymocytes and peripheral T cells. Based on its inducible enhancer activity in Jurkat cells and inducible histone acetylation in thymocytes, CNS4 appears to function as a thymocyte enhancer as well. Thus, we suggest that CNS4 may not only function as an enhancer in the context of the endogenous CD69 locus, but may, perhaps in conjunction with CNS3, function as an antisilencer that switches CNS2 from silencer to enhancer activity. We propose that all four CNSs may converge to interact physically and functionally in the form of an active chromatin hub, as initially described for the β-globin locus (44). Perhaps consistent with this, a previous study suggested that CD69 may be regulated by the architectural protein SATB1 (45).

We note that our conclusions about the activities of CNS1–4 in vivo assume that these elements function normally in the context of our relatively compact Tg reporter. Prior studies have validated this approach in other systems (18, 46). However, we cannot rule out that our Tg constructs lack relevant elements from DNA segments that normally separate the CNSs or cannot adopt important three-dimensional chromatin configurations that are important for physiological regulation. To further study mechanisms of CD69 regulation in vivo we will analyze mice transgenic for a bacterial artificial chromosome containing the mCD69 locus.

As compared with thymocytes, the mechanism of induction of CD69 gene expression in mature T cells appears distinct, as only CNS1 displayed inducible H3 histone acetylation and H3 K4 dimethylation in the latter cell population. This was true despite the fact that, as for thymocytes, CNS2–4 were needed for tightly regulated, inducible expression in peripheral T cells of Tg mice. We

suggest that CNS2–4 activity during T cell development may establish a specific chromatin context that is required for proper regulation of the CD69 locus in mature T cells.

Although the combination of all four CNSs was capable of promoting high-level and tightly regulated reporter gene expression in thymocytes and peripheral T lymphocyte populations, it did not do so in all Tg lines. One explanation for this is that additional elements may be required to reconstitute a CD69 locus control region that can consistently overcome chromosomal position effects. However, we note that hCD2 expression was both low and variegated in those Tg lines containing particularly high copy reporter arrays. Thus, it is possible that RNA interference may cause transgene silencing in these lines (47).

The unexpected finding that Tg mice with all CNSs misexpressed the hCD2 reporter in unstimulated B cells indicates that CD69 gene transcription is differentially controlled in B and T cells. Thus, it seems likely that the construct lacked an element required to repress the expression in unstimulated B cells. Further evidence for differential regulation in T and B cells was obtained from the epigenetic profile of CD69, which revealed distinct patterns of histone modifications at CNS2, CNS3, and CNS4 between T and B cells. In this regard, IL-2 transcription is thought to be regulated by different mechanisms in CD4 and CD8 lymphocytes, and IL-4 and IL-13 transcription is reduced in Th2 cells but not in mast cells in mice deficient for the CNS1 region (48, 49). Interestingly, Tg mice bearing a 30-kb genomic fragment containing Ly49A, another gene in the NK complex that encodes a C-type lectin, also showed aberrant expression in B cells. Perhaps similar mechanisms are used to suppress CD69 and Ly49A expression in B cells (50).

Based on our findings, we propose that the CD69 promoter and upstream elements display an accessible chromatin structure before CD69 transcription to support rapid gene induction upon stimulation. Gene induction requires the combined activity of multiple upstream CNSs, two of which display classical enhancer activity in T cells. The interdependence of these CNSs may also allow for physiological repression of CD69 expression by disrupting CNS3 and CNS4 interactions with CNS1 and CNS2. Finally, an as yet uncharacterized B cell-specific element may be required to suppress CNS activity in unstimulated B cells. The above results indicate that CD69 gene regulation is complex and likely differs in different cell types. Future studies are required to elucidate the nature of CD69 regulatory elements and the mechanism through which they regulate CD69 expression.

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Disclosures

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